博士論文

# The miR-155-5p inhibits osteoclast differentiation through targeting CXCR2 in orthodontic root Resorption

（マイクロRNA155-5pは矯正学的歯の移動に伴う歯根吸収での破骨細胞分化をCXCR2を標的として阻害する）

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**Abstract**

Root resorption is an unavoidable side effect of orthodontic tooth movement. The mechanism of root resorption is similar to bone resorption, the odontoclasts shares similar characteristics with osteoclasts (OCs). MicroRNAs (miRNAs) such as miR-155-5p play an important role in OC differentiation, but the underlying molecular mechanism of miR-155-5p in this process is not fully understood. We found that the miR-155-5p seed sequences were complementary to a sequence conserved in the 3-untranslated region of CXCR2 mRNA. In this study we explored the molecular mechanism underlying the effect of miR-155-5p on OC differentiation by targeting CXCR2. In this study, we divided the orthodontic patients into mild, moderate and severe groups according to the severity of root resorption. The gingival crevicular fluid (GCF) of patients in different groups was collected, and the expression levels of dentin phosphoprotein (DPP) were detected by ELISA, and the expression levels of CXCR2 and miR-155-5p in GCF were detected by real-time quantitative PCR (qRT-PCR). The relationship between miR-155-5p and CXCR2 was verified by double luciferase. We analyzed changes of CXCR2 and miR-155-5p expression after transfection of miR-155-5p mimic and inhibitor into RAW264.7 cells induced by receptor activator of nuclear factor-κB ligand (RANKL) through qRT-PCR and western blotting. The effect of miR-155-5p on OC differentiation was evaluated by tartrate resistant acid phosphatase (TRAP) staining. QRT-PCR and western blotting were used to analyze expression of the osteoclastic bone resorption-related enzymes CA II, MMP-9 and cathepsin K. To further confirm the direct targeting effect of CXCR2 by miR-155-5p, we blocked CXCR2 using si-CXCR2 in RANKL-induced RAW264.7 cells. DPP levels were consistent with the trend of CXCR2 changes, and the trend of CXCR2 expression was opposite to miR-155-5p changes. miR-155-5p can be directly targeted to act on CXCR2. The expression

of miR-155-5p was significantly downregulated in differentiated OCs. MiR-155-5p inhibited OC differentiation, and downregulated CA II, MMP-9 and cathepsin K expression at the protein and mRNA levels.

In summary, the results of this study suggested that miR-155-5p inhibited OC differentiation by targeting CXCR2, thus reducing root resorption in orthodontics. MiR-155-5p can be used as an effective target for avoiding or reducing the degree of root resorption in orthodontic treatment.

**Keywords:** miR-155-5p, Osteoclast differentiation, RANKL, CXCR2

## Introduction

Root resorption is a common sequela of orthodontic tooth movement.[1] Root resorption caused by orthodontics seriously affects the effect of orthodontic treatment, and how to avoid root resorption of orthodontic is a research hotspot in orthodontic treatment. Root resorption is a process in which the cementum or dentin layer is destroyed by osteoclasts (OCs), which leads to a defective root surface structure. There are two major types of root resorption: internal resorption and external resorption, and the root resorption caused by the orthodonticis external resorption[2].

The degree of orthodontic derived root resorption can be divided into four grades according to the severity of root resorption, the mild resorption (grade 1): root with its normal length, and only an irregular contour; the moderate resorption (grade 2): the absorption area was small, and the tip of the root tip has a nearly straight profile; the accentuated resorption (grade 3): loss of nearly one third of root length; extreme resorption (grade 4): Loss of more than a third of the root length of the tooth[3]. It is the progressive absorption of the apical hard tissue, which shortens the root obviously[4].The progressive resorption of the apical shows that the root resorption is serious and the root is shortened obviously by X-ray, which is one of the serious complications of orthodontic treatment. Brezniak et al. described root resorption during orthodontic treatment as orthodontically-induced inflammatory root resorption (OIIR).[4] In the orthodontic process, the mineralized cementum was exposed by mechanical pressure, which attract the TRAP-positive odontoclast adhesion to degrade minerals and organic matter in cementum, and forms a large number of Howship's lacunae, and finally fuses into a wide range of root resorption area.[5] OIIRR is a highly complex process, which occurs at the same time as remodeling of the periodontal ligament and alveolar bone.[4] Orthodontic treatment can lead

to hyaline degeneration of periodontal tissue, and root resorption occurs on the root surface adjacent to the hyaline degeneration area.[6] Precursors OCs enter the periodontal ligament from the bone marrow cavity of alveolar bone during OIIRR, destroying the cementum and dentin while resorbing hyaline and alveolar bone, until the hyaline tissue is completely removed.[7,8] OCs are terminally-differentiated multinucleated cells formed by the fusion of monocyte precursors, OCs attach to the bone matrix during bone resorption, reduce extracellular pH to dissolve the hydroxyapatite, and secrete proteolytic enzymes, including cathepsin K and matrix metalloproteinase-9 (MMP-9) to digest the organic matrix.[9] RANKL and osteoprotegerin (OPG) are the most important factors in OC differentiation.[10] Osteoblasts and stromal stem cells express RANKL, which binds to RANK on the surface of OCs and their precursor cells, thus regulating the normal activation and differentiation of precursor cells into polynuclear-OCs. OPG, as a water soluble tumor necrosis factor receptor molecules, by inhibiting the frilled edge of mature osteoclast formation, amd has become the natural inhibitors of RANKL, it can combine with RANKL, inhibit the interaction between RANKL/RANK, regulating and inhibiting bone resorption.[11] Under normal conditions, the activity of osteoblasts and OCs is in a state of dynamic balance, thus maintaining the physiological health of the root and surrounding bone tissue.[12] If this balance is disrupted, the tooth-bone and dentin are removed, resulting in root resorption. It has been found that in severe root resorption, there is a high level of RANKL secretion in compressed periodontal ligament cells,[13] and OPG decreased production.

Gingival crevicular fluid (GCF) is an extracellular fluid secreted from the epithelia of the gingival crevice,[14] it is an inlfammatory exudate within the gingival tissues and flows into the gingival sulcus continually. Laura Balducci et al found that the concentration of DPP in the GCF was significantly higher in the orthodontic root resorption group than in the non-resorption group,[15] and therefore speculated that DPP might be a biochemical marker

for detecting root resorption.

Orthodontic tooth movement is the result of a series of responses by periodontal tissue cells to biomechanical stimuli, this process was a sterile inflammatory response. In this process, the migrated immune cells, together with natural cells such as fibroblasts and osteoblasts, produce inflammatory cytokines, such as growth factors, chemokines, colony stimulating factors, etc.[16,17] According to the difference of N-terminal cysteine residues, chemokine can be divided into five subfamiles, CXC, CC, XC, CX3C and CX. [18]Chemokines interact with their receptors to make the target cells chemotaxis and migrate, enhance the adhesion between the target cells and endothelial cells, and enhance the physiological functions such as the formation, differentiation and apoptosis of the target cells.[19] Chemokine is a large family, which provides a key signal for the transport, differentiation and activity of osteocytes.[20] It has been reported that IL-8 and MCP-1 may promote the process of root resorption during orthodontics.[21] IL-8 is a potent inflammatory cytokine with multiple biological functions, which plays an important role in chemotaxis and activation of neutrophils in the process of inflammation.[22] During orthodontic tooth movement process, the changes of IL-8 content in GCF reflected the inflammatory state of periodontal tissue and bone resorption in the corresponding area.[23] CXCR2 is one of the receptors of IL-8, which exists on the surface of macrophages, epithelial cells, osteoblasts and OCs.[24] CXCR2 is one of the G protein-coupled receptors and participates in a variety of inflammatory processes.[25]

MiRNAs are a type of endogenous single-stranded non-coding small RNA, which exist widely in the biological genome, and can degrade or inhibit mRNA translation by partially-complementary pairing with target mRNA 3'UTRs.[26] MiRNAs play an important role in the differentiation and formation of OCs. Sugatani et al. reported that the complete loss of miRNA activity in OC precursors promoted the formation of OCs.[27] The specific

loss of key enzymes involved in miRNA biosynthesis in mononuclear OC precursors and mature multinucleated OCs leads to a decrease in the number and activity of OCs, which in turn leads to an increase in bone mass.[28,29] The role of miR-155 in OC differentiation has been reported in recent years. Mann et al. found that the expression of miR-155 increased significantly during differentiation of RAW264.7 cells into macrophages, but decreased significantly during OC differentiation.[30] Zhang et al. proved that miR-155 plays an important role in the mechanism of OC differentiation induced by interferon β (IFN-β), and down-regulated OC formation by inhibiting cytokine signal transduction inhibitor protein 1 (SOCS1) and microphthalmia (MITF).[31]

In our study, we found that miR-155-5p directly targeted CXCR2 through bioinformatics prediction and luciferase activity determination. We speculate that miR-155-5p can inhibit osteoclast differentiation through targeted regulation of CXCR2 expression during orthodontic root resorption. In order to confirm the interaction between CXCR2 and miR-155-5p in OC differentiation, we established an in vitro model using RANKL-induced RAW264.7 cells to explore the molecular mechanism of the effect of miR-155-5p on OC differentiation by targeting CXCR2.

## Materials and methods

### Patients

There were 60 orthodontic patients, including 28 males and 32 females, aged from 27 to 71 years, with an average of (46.5 ±6.7) years. According to the severity of root resorption, it was divided into mild (grade1, n=19), moderate (grade2, n=26) and severe (grade 3-4,n=15). There were 20 healthy controls, including 9 males and 11 females, aged from 30 to 70 years, with an average of (46.0 ±7.0) years. The x-rays of the samples we collected in our study were provided in the supplemental files. This study was approved by the Ethics Committee of Dalian Stomatological Hospital and was performed under the informed consent of the patients.

### Gingival crevicular fluid (GCF) sampling

The 3# Whatman filter paper (Whatman, Maidstone, UK) was cut into small strips of 2mm × 8mm. Every 8 strips were packed in an EP tube, weighed, sterilized at high temperature and high pressure, then dried and set aside. The patient's teeth were cleaned with alcohol-soaked cotton balls before sampling, and then use cotton balls to insulate the moisture and blow-dry the tooth surface. The filter paper strips were gently placed into the buccal and lingual gingival sulcus of the proximal and distal teeth, stopped in case of slight resistance, and removed after 30s. If the moisture-absorbing paper tip was stained with blood or saliva, it was discarded and repeated twice after 2min interval. The filter paper strips adsorbing gingival crevicular fluid were sealed in the EP tube, weighed again, frozen and stored in the refrigerator at-70 ℃.

Elisa for DPP

Dentin phosphoprotein (dentin phosphoproteins,DPP) is a tooth-specific protein derived from dentin sialophosphoprotein (dentin sialophosphoprotein,DSPP). In this study, DSPP was measured to reflect the expression of DPP. The frozen samples were taken out and balanced to room temperature. The 200 ul phosphate buffer solution（0.01mol/L, pH = 7.4） was added to each EP tube, centrifuged at 3000g,15min, at 4° C. According to the dentin sialoprotein quantitative (DSPP) EIASA kit (USBiological, MA, USA) instruction to operation, and the OD values were measured at 450nm.

Cell culture

The RAW264.7 cell line was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured in DMEM containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 μg/mL of penicillin and 100 μg/mL of streptomycin (Gibco, Carlsbad, CA, USA). The concentration of RANKL (R&D Systems Ltd., Minneapolis, MN, USA) required to induce RAW264.7 cells to differentiate into OCs was 50 ng/mL.

Transfection with miR-155-5p inhibitor or mimic

Cell transfections were performed using the liposome transfection kit Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). According to the manufacturer's instructions, the RAW264.7 cells were inoculated into a 6-well plate at a density of 2 × 105 cells/mL. After reaching 50% confluence, RAW264.7 cells were transfected with 20 nM miR-155-5p mimic, mimic-con, miR-155-5p-inhibitor or inhibitor-con. The plasmids were synthesized by GenePharma Co. (Shanghai, China). Cells were cultured in DMEM

containing 10% FBS, 50 ng/mL RNAKL, 100 μg/mL penicillin and 100 μg/mL streptomycin in an environment of 5% CO2, 37°C and 95% humidity for 3d. The morphological changes of RAW264.7 cells were observed by inverted light microscope (Nikon, Chiyoda-ku, Japan). The sequences of mimic -con, mimic, inhibitor -con, and inhibitor were shown in table 1.

The miRNA target prediction and dual luciferase reporter assay

In this study, miranda and TargetScan were selected as the miRNA target gene prediction tools. The prompts of the data website were followed to predict the potential targets of miR-155-5P. To perform a dual luciferase reporter assay, RAW264.7 cells were inoculated into 24-well plates at a density of $2 \times 10^4$ cells per well. Cells were cultured overnight, then once cell adhesion was observed, the culture medium was changed to remove nonadherent cells, and the medium was changed to fresh medium. A Lip2000 transfection kit was used to co-transfect the firefly luciferase gene plasmid pGL3-CXCR2-wt-3'UTR/pGL3-CXCR2-mut-3'UTR and miR-155-5p mimic/con plasmid. The renilla luciferase plasmid pRL-TK was co-transfected to normalize for transfection efficiency. After 48 hours of transfection, according to the manufacturer's instructions, the activity of luciferase was measured using a dual luciferase reporter system. Each sample was assayed in triplicate.

Small interfering RNA (siRNA) transfection

RAW264.7 cells transfected with different plasmids were inoculated into 6-well plates at a density of $5 \times 10^5$/mL cells per well. The cells were cultured overnight, then once cell adhesion was confirmed, the culture medium was changed to remove the nonadherent cells. With the exception of the control group, all the other groups were cultured in medium

containing the cytokine RANKL for 24 hours. The liposome transfection method was used to transfect siRNAs, using a transfection kit purchased from Cell Signaling Technology (Danvers, MA, USA) according to the manufacturer's instructions. The concentration of the double-stranded siRNA transfection plasmid was 20 μM, and transfection was performed in culture medium without fetal bovine serum or antibiotics. After transfection, cells were cultured in medium containing RANKL for 24 hours. The sequences of si-con and si-CXCR2 used were shown in table 1.

Real-time quantitative PCR assay

RANKL-induced RAW264.7 cells transfected with different plasmids were inoculated into 6-well plates at a density of $5 \times 10^5$/mL cells per well. The cells were cultured overnight, and when cell adhesion was observed, the culture medium was changed to remove the nonadherent cells. Apart from the control group, all the other groups were cultured in medium containing RANKL for 24 hours. RNA was extracted using TRIzol (Invitrogen). A PrimeScript reverse transcriptase kit and SYBR Premix ExTaq kit were purchased from Takara Bio Inc. (Shiga, Japan), and PCR primers were synthesized by TAKARA BIO INC (JPN). All samples were tested three times independently and the relative expression levels were calculated using the 2-△△Ct method. The sequence were listed as following: ( mouse CXCR2 : forward ATGCCCTCTATTCTGCCAGAT; reverse GTGCTCCGGTTGTATAAGATGAC; MMP-9: forward GCGTCGTGATCCCCACTTAC, reverse CAGGCCGAATAGGAGCGTC ; Cathepsin k :forward GAAGAAGACTCACCAGAAGCAG22, feverse TCCAGGTTATGGGCAGAGATT; CA II forward GATAAAGCTGCGTCCAAGAGC, reverse GCATTGTCCTGAGAGTCATCAAA; GAPDH forward

TCAAGATCATCAGCAATGCC, reverse CGATACCAAAGTTGTCATGGA; Human CXCR2: forward TTTCATAGGTCACAGCTGCT, reverse GAGGTAAACTTAAATCCTGACTGG, GAPDH: 5′- TCGTGGAAGGACTCATGACC-3′ , Reverse GAGGCAGGGATGATGTTCTG-3′).

Western blot assay

RANKL-induced RAW264.7 cells transfected with different plasmids were inoculated into 6-well plates at a density of $5 \times 10^5$/mL cells per well. The cells were cultured overnight to allow cell adhesion, then the culture medium was changed to remove the non-adherent cells. Apart from the control group, all the other groups were cultured in medium containing RANKL for 24 hours. Total protein was extracted from cells using RIPA lysis buffer (Beyotime, Shanghai, China), and the proteins were quantitatively determined by the Bradford method (Beyotime). Aliquots containing 30 μg total protein were separated by 10% SDS-PAGE gel electrophoresis. The separated proteins were then transferred onto PVDF membranes (Thermo Fisher Scientific). The PVDF membranes were blocked in TBST containing 5% skimmed milk for 1 hour, and then incubated with specific primary antibodies at 4°C overnight. Next day, the PVDF membranes were rinsed with TBST three times at room temperature, 10 min per time. After rinsing, the PVDF membranes were incubated at room temperature with the secondary antibody for 2 hours. Then, the PVDF membranes were rinsed with TBST three times at room temperature, 10 min per time, and labeled proteins were detected using Immobilon WesternChemilu miniature HRP substrate (Merck-Millipore, Darmstadt, Germany), and protein expression was quantitatively analyzed using ImageJ gel analysis software. The antibodies CXCL8 (Rabbit polyclonal, Invitrogen, Carlsbad, CA, USA, PA5-114316, 1:1000 dilution), CXCL12 (Rabbit polyclonal, Invitrogen,

Carlsbad, CA, USA, PA5-17238, 1:500 dilution), CXCR1 (Rabbit polyclonal, Invitrogen, Carlsbad, CA, USA, PA1-29466, 1:800 dilution), CXCR2 (Rabbit polyclonal, Abcam, Cambridge, MA, USA, PA5-94935, 1:1000 dilution), CXCR3 (Rabbit polyclonal, Invitrogen, Carlsbad, CA, USA, PA5-88164, 1:500 dilution), CXCR4 (Rabbit polyclonal, Invitrogen, Carlsbad, CA, USA, PA5-105597, 1:300 dilution), CXCR5 (Rabbit polyclonal, Invitrogen, Carlsbad, CA, USA, PA5-100726, 1:800 dilution), Carbonic anhydrase 2 (CA II) (Rabbit polyclonal, Invitrogen, Carlsbad, CA, USA, PA5-51598, 1:1000 dilution), MMP-9 (Rabbit polyclonal, Invitrogen, Carlsbad, CA, USA, PA5-13199, 1:500 dilution), and Cathepsin K (Rabbit polyclonal, Invitrogen, Carlsbad, CA, USA, PA5-109605, 1:800 dilution), as well as HRP-secondary antibodies (goat anti-rabbit, Invitrogen, Carlsbad, CA, USA, 1:3000 dilution). All data analyses were independently repeated for three times.

Tartrate resistant acid phosphatase (TRAP) staining assay

RANKL-induced RAW264.7 cells transfected with different plasmids were inoculated into 6-well plates at a density of $5 \times 10^5$/mL cells per well. The cells were cultured overnight to allow them to adhere, then the culture medium was changed to remove the nonadherent cells. Apart from the control group, all the other groups were cultured in medium containing RANKL for 5 days. After 5 days of induction, the cells were stained using a TRAP staining kit purchased from Sigma-Aldrich (St. Louis, MO, USA) according to the manufacturer's instructions. OCs were identified as TRAP-positive, multinucleated cells, containing three or more nuclei. Each experiment was performed in triplicate. The original pictures of low magnification and high magnification in the supplementary materials.

TRAP activity assay

After 5 days of incubation, the culture medium was discarded and washed lightly with PBS for 3 times, then the absorbance value of the formation of p-nitrophenol were measured at 405 nm according to the instructions of TRAP assay kit (Sigma-Aldrich, St . Louis MO, USA) by Elx808 microplate reader (Bio-Tek InstrUSAnts Inc., USA). Each experiment was performed in triplicate.

Statistical analysis

The software used for statistical analysis in this study was SPSS 20.0 (IBM SPSS Statistics for Windows, IBM, Armonk, NY, USA). The measurement data from all experiments are expressed as mean ± SD; Student's *t*-test and one-way analysis of variance (ANOVA) were used to assess group differences. Differences were considered statistically significant when $P < 0.05$. All experiments in this study were repeated more than three times.

## Results

The expression of DPP, CXCR2 and miR-155-5p in patients with different degrees of orthodontic root resorption

In this study, we detected the expression of DPP, CXCR2 and miR-155-5p in GCF of orthodontic patients with different degrees of root resorption. The results showed that the expression of CXCR2 and DPP increased significantly with the increase of the severity of root resorption, and there was significant difference among patients with different degrees of root resorption (Fig.1 A and C, $P < 0.05$). The expression of miR-155-5p decreased significantly with the increase of the severity of root resorption (Fig.1B, $P < 0.05$), and the change trend of miR-155-5p was negatively correlated with CXCR2 ($r = 0.511$, $P < 0.001$).

CXCR2 is a target gene of miR-155-5p

In order to explore whether CXCR2 was a target gene of miR-155-5p, the miRNA target gene prediction tools Miranda and Targetscan database were used. The results showed that the 3'-UTR of CXCR2 contained a conserved miR-155-5p binding site (Fig. 2A). To confirm that CXCR2 was the target gene of miR-155-5p, we used a dual luciferase reporter assay to construct luciferase reporter plasmids of the wild-type and mutant 3'-UTR of CXCR2. At the same time, we also designed miR-155-5p mimic and mimiccon plasmids. MiR-155-5p mimics/mimiccon and target gene luciferase reporter plasmids were co-transfected into RAW264.7cells. The results showed that the luciferase activity of cells co-transfected with CXCR2-wt and miR-155-5p mimics decreased significantly (Fig.2A, $P < 0.05$), while the luciferase activity of cells co-transfected with CXCR2-mut and miR-155-5p mimics showed no significant change compared with the control group (Fig.2A, $P > 0.05$). We also examined the expression of miR-155-5p in the different intervention groups and found that the expression of miR-155-5p in RNAKL- RAW264.7cells was significantly decreased compared to the control group (Fig.2B, $P < 0.05$); miR-155-5p expression was significantly increased after transfection of miR-155-5p-mimic (Fig.2B, $P < 0.05$); miR-155-5p expression was significantly increased compared to miR-155-5p-inhibitor group (Fig.2B, $P < 0.05$); the expression of miR-155-5p was significantly reduced compared to miR-155-5p-con group (Fig.2B, $P < 0.05$). In order to further confirm our findings, we investigated the effect of miR-155-5p on CXCR2 expression in RANKL-induced RAW264.7 cells by RT-PCR and western blotting. Compared with the cells transfected with the miR-155-5p-con, the miR-155-5p mimics inhibited the expression of CXCR2 protein and mRNA in RANKL-induced RAW264.7 cells. The expression of CXCR2 protein and mRNA in RAW264.7 cells transfected with miR-155-5p inhibitor plasmid was significantly higher than that in cells with negative control (Fig.2C, $P < 0.05$). In order to further verify that

miR-155-5p directly regulates the expression of CXCR2, we analyzed the expression of CXCR1, CXCR3, CXCR4, CXCR5, CXCL8 and CXCL12. The results showed that the change trends of CXCL8, CXCL12 were similar to that of CXCR2, but the expression of CXCR1, CXCR3, CXCR4 and CXCR5 were all upregulated, and their expression was not affected by the expression of miR-155-5p (Fig.2D). These results suggested that miR-155-5p directly inhibited the translation and expression of CXCR2 in RANKL-induced RAW264.7 cells.

The miR-155-5p inhibits RANKL-induced osteoclast differentiation

We found that miR-155-5p inhibited the translation and expression of CXCR2 in RANKL-induced RAW264.7 cells. In order to further confirm the effect of miR-155-5p on OC differentiation, we used TRAP staining to detect the formation of OCs. RANKL-induced RAW264.7 cells were transfected with different plasmids. The results showed that transfection with miR-155-5p mimic significantly inhibited OC formation, while after transfection with miR-155-5p inhibitor plasmid, OC formation was significantly increased ($P<0.05$, Fig. 3A–C). There is no difference in number of OCs or in TRAP activity between RANKL and mimic-con/inhibitor-con ($P>0.05$, Fig. 3A–C). In order to further support our view that miR-155-5p inhibits OC differentiation, we used RT-PCR and western blotting to analyze the mRNA and protein expression of the osteoclastic bone resorption-related enzymes CA II, MMP-9 and cathepsin K. The results showed that both the mRNA and protein expression of CAII, MMP-9 and cathepsin K were inhibited in miR-155-5p mimic-transfected cells. At the same time, the expressions of CAII, MMP-9 and cathepsin K mRNA (Fig. 4A) and protein (Fig. 4B) in miR-155-5p inhibitor-transfected cells were significantly increased ($P<0.05$). These results suggested that miR-155-5p inhibited the

expression of bone resorption-related enzymes in OCs.

CXCR2 silencing weakened miR-155-5p inhibition of osteoclast differentiation

In order to fully confirm the role of CXCR2 in miR-155-5p-mediated inhibition of RANKL-induced OC differentiation, we used the siRNA transfection technique to silence the CXCR2 of RANKL-induced RAW264.7 cells. We first analyzed the efficiency of siRNA using western blotting; the results showed that CXCR2 siRNA significantly reduced the expression of CXCR2, but after transfection of miR-155-5p inhibitor, the expression of CXCR2 was significantly increased(Fig. 5A). Meanwhile, the expression of CXCR2 after transfection of CXCR2 siRNA and miR-155-5p inhibitor was not significantly different compared with the control group. Similarly, in order to verify that the expression of CXCR2 was directly regulated by miR-155-5p, we analyzed the expression of CXCR1, CXCR3, CXCR4, CXCR5, CXCL8 and CXCL12. The results showed that the change trends of CXCL8 and CXCL12 were similar to CXCR2, but the changes in the expression of CXCR1, CXCR3, CXCR4 and CXCR5 were not significant and were not affected by the expression levels of CXCR2 or miR-155-5p (Fig. 5B). Through TRAP staining, we found that OC differentiation was significantly inhibited after transfection of CXCR2 siRNA, while OC formation was significantly increased after transfection of miR-155-5p inhibitor; and transfection of both CXCR2 siRNA and miR-155-5p inhibitor led to no significant difference compared with the control group (Fig. 5C–E). Similarly, we used RT-PCR and western blotting to analyze the expression of bone resorption-related enzymes in OCs under silenced expression of CXCR2 (Fig. 6A–B). The results showed that compared with the control group, the mRNA and protein expression of CAII, MMP-9 and cathepsin K were all inhibited after CXCR2 siRNA transfection into RANKL-induced RAW264.7 cells. In

contrast, the expression of mRNA and protein of CAII, MMP-9 and cathepsin K increased after miR-155-5p inhibitor transfection, while transfection of both CXCR2 siRNA and miR-155-5p inhibitor produced no significant change in the expression of mRNA and protein of CAII, MMP-9 and cathepsin K compared with the control group. The results suggested that miR-155-5p induced OC differentiation by targeting CXCR2 to inhibit RANKL.

## Discussion

The early stages of orthodontic tooth movement is an acute inflammatory process, which is characterized by periodontal vasodilation and leukocyte migration from capillaries.[32] Wehrbein et al. found that orthodontic treatment can easily lead to root resorption,[33] orthodontic root resorption is a process of destruction, degradation and reconstruction of root tissue (cementum and dentin), root resorption and deep periodontal tissue changes, as well as the degradation of non-collagen dentin and cementum protein and the release of destruction products can fluid into GCF, and lead to the composition of GCF changed. In this study, we detected the changes of DPP, CXCR2 and miR-155-5p in GCF of orthodontic patients with different degrees of root resorption. DPP is an indicator of root resorption.[15] We found that the expression of DPP was consistent with that of CXCR2, but opposite to that of miR-155-5p. At the same time, the change of miR-155-5p was negatively correlated with CXCR2.

Cementoclasts are similar to OCs, with similar morphology and biological function, are derived from similar precursor cells,[34] have similar enzymatic and metabolic properties,[35] and are regulated by the same mechanism as bone resorption.[36] OCs specifically express TRAP, and attach to the bone surface to secrete acidic substances and proteases to dissolve the bone.[37] Cathepsin K and CA II are related to the bone resorptive function of mature OCs,[38] and the expression of cathepsin K and CA II is necessary to bone resorption. In the process of root resorption, the process of cementum destruction by cementum cells mainly includes two biological processes: acidification and degradation of organic matrix. Cathepsin K is a new cysteine protease found in osteoclasts, it is considered to be the most important and highly expressed proteolytic enzyme involved in OC activity,[21] which acts in the degradation of organic matter together with other proteolytic enzymes in the process of bone

resorption. CK mRNA were expressed in osteoclasts in root resorption lacunae, osteoclasts in bone resorption lacunae and periodontal ligament fibroblasts.[39] CK is synthesized and secreted into the root resorption lacunae by osteoclast,[40] CK not only participates in the degradation of dentin matrix, but also participates in the degradation of lysosome in vivo. Matrix metalloproteinases play an important role in the development, repair and inflammation of almost all kinds of tissues, as well as in normal bone remodeling and bone resorption.[41] Domon et al. believe that proteases secreted by OCs are directly involved in the degradation of cementum organic matrix, including cathepsin K and matrix metalloproteinases (MMPs).[42] Ohba et al. reported that a small number of cathepsin K mRNA-positive OCs could be detected in the pressure area of periodontal tissue after tooth stress.[43] MMP-9 is an extracellular protease highly expressed in OCs, which plays an important role in bone resorption and participates in cell migration, some studies have found that MMP-9 is expressed on the OCs absorbed by the root of bovine deciduous teeth,[44] and MMP9 is also significantly expressed in orthodontic root resorption.

The RAW264.7 cell line used in this study is a good OC progenitor cell model.[45] This cell line represents the relatively late stage of differentiation from hematopoietic stem cells (HSCs) to mature OCs. Induction by RANKL provides the necessary conditions for inducing mature OCs, and a large number of mature OCs can be obtained. RANKL, a member of the (TNFR) family of tumor necrosis factor receptors, can be secreted by OCs, and its expression is regulated by a variety of cytokines and hormones.[46] We found that RANKL up-regulated the expression of CXCR2 when stimulating the differentiation and maturation of osteoclast cell line RAW264.7, which indicated that RANKL had a positive feedback regulation on IL-8/CXCR2 signal pathway. Therefore, CXCR2 may play an important role in the maturation and differentiation of osteoclasts mediated by RANKL. It has been found that CXCR2 is involved in a variety of pathological processes, including chronic

inflammation, pathological osteolysis and atherosclerosis.[47] CXCR2 has been shown to be involved in a variety of pathological processes, including chronic inflammation, pathological osteolysis and atherosclerosis.[48] A study by Wang et al. revealed that CXCR2 plays a key role in particle-induced osteolysis.[47] Weiland et al. reported that root resorption induced by orthodontic force involves a series of aseptic inflammatory processes.[48] Some studies have shown that inflammatory factors such as IL-1, IL-6, IL-8 and TNF-α are expressed in root resorption,[49] and play a certain role in promoting root resorption. In orthodontics, root resorption has aseptic inflammatory characteristics, We infer that CXCR2 plays an important role in the regulation of osteoclast differentiation in root resorption.

MiR-155-5P is an inflammation-associated miRNA that regulates inflammation and immune cell function at multiple levels.[50] In this study, we found that miR-155-5p could directly target CXCR2. Before study reported that the expression level of miR-155 in murine bone marrow macrophages (BMMs) is upregulated by TNF-α/RANKL/M-CSF treatment, but is slightly downregulated by RANKL/M-CSF treatment during osteoclast formation .[51] Similarly, Mann et al.[30] showed that during the differentiation of osteoclasts, the expression level of miR-155 was up-regulated when macrophages were activated, but decreased significantly in osteoclasts. Zhao et al. also found that miR-155 can inhibit osteoclast differentiation.[52] We have come to the same conclusion in our study. In RANKL-induced RAW264.7 cells transfected miR-155-5p mimic and inhibitor, we found that. MiR-155-5p can regulate the expression of CXCR2 at both protein and mRNA levels. By analyzing the expression of other CXCR family members, CXCL8 and CXCL12, CXCL8 was the ligands of CXCR2. We found that the expression status of miR-155-5p and CXCR2 did not affect the expression of other cytokines. Some findings demonstrate that miR-155 regulates osteoclastogenesis via targeting several essential transcriptional factors.[53] In this study, we speculated that miR-155-5p binding directly inhibited the expression of CXCR2. It is

suggested that miR-155-5p could inhibit OC differentiation by inhibiting CXCR2 expression. We found miR-155-5p overexpression can reduced the formation of TRAP-positive multinucleated cells, and reduced the expression of CA II, MMP-9 and cathepsin K. Correspondingly, inhibition of miR-155-5p expression increased the number of TRAP-positive multinucleated giant cells and enhanced the CA II, MMP-9 and cathepsin K expression. The results were similar when CXCR2 was silenced. Based on the above results, we believe that miR-155-5p inhibits OC differentiation, and this inhibitory effect was achieved by targeting CXCR2.

Based on the above studies, we speculate that in OIIRR, miR-155-5p inhibited OC differentiation by directly targeting CXCR2. First, we used miRNA target online prediction tools to predict the target of miR-155-5p, and found that CXCR2 was the direct target of miR-155-5p. In order to verify this discovery, we employed the luciferase activity assay, and the results confirmed that miR-155-5p can directly target CXCR2.

To sum up, this study found that miR-155-5p could reduce the expression of CXCR2 and inhibit OC differentiation by targeting CXCR2, thus reduced root resorption degrees in orthodontics. MiR-155-5p can be used as an effective target for avoiding or reducing the degree of root resorption in orthodontic treatment.

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**Figure Legends**

**Fig. 1. The expression of DPP,CXCR2,miR-155-5p in GCF.** (A)The DPP expression level was detected by ELISA.(B,C)The CXCR2 and miR-155-5p expression levels were detected by qRT-PCR.(D)The expression relation between miR-155-5p and CXCR2.Data is presented as the mean ± SD of three independent experiments.The data represent three experiment exhibiting similar results. # p<.05 versus healthy group; * p<.05 versus mild group;@p<.05 versus moderate group.

**Fig. 2. CXCR2 is a target of miR-155-5p in RAW264.7 cells.** (A) The renilla luciferase activity was determined by dual luciferase reporter system. (B) The miR-155-5pexpression levels were detected by qRT-PCR. (C) CXCL8,CXCL12,CXCR1-5,and GAPDH were detected by western blotting. Data is presented as the mean ±SD of three independent experiments.The data represent three experiment exhibiting similar results. #p<.05 versus control(-RANKL); *p<.05 versus mimic-con(+RANKL); @p<.05 versus inhibitor-con(+RANKL). RANKL,receptor activator of nuclear factor-kB ligand.

**Fig. 3. The miR-155-5p inhibited osteoclastogenesis in RAW264.7 cells.**(A)The formation of osteoclasts was detected by TRAP staining. (B) TRAP-positive cell count. (C) TRAP activity. Data are presented as the mean ± SD of three independent experiments.The data represent three experiment exhibiting similar results. # p<.05 versus control(-RANKL); * p<.05 versus mimic-con(+RANKL); @ p<.05 versus inhibitor-con(+RANKL). RANKL,receptor activator of nuclear factor-kB ligand; TRAP,tartrate-resistant acid phosphatase.

**Fig. 4. The miR-155-5p inhibited the expression of osteoclastic bone resorption-related enzymes in RAW264.7 cells.**(A)The bone resorption-related enzyme mRNA expression levels were detected by RT-PCR. (B) The bone resorption-related enzyme protein expression levels were detected by western blotting.Data are presented as the mean±SD of three independent experiments.The data represent three experiment exhibiting similar results. # p<.05 versus control(-RANKL); * p<.05 versus mimic-con(+RANKL); @p<.05 versus inhibitor-con(+RANKL). RANKL,receptor activator of nuclear factor-kB ligand.

**Fig.5.The si-CXCR2 weakened the promotional effect of miR-155-5p inhibitor on osteoclastogenesis in RAW264.7 cells.** ① si-con+inhibitor-con; ② si-con+inhibitor; ③ si-CXCR2+inhibitor-con; ④ si-CXCR2+inhibitor.(A-B) The CXCL8, CXCL12, CXCR1-5, and GAPDH were detected by western blotting. (C-E) The formation of osteoclasts was analyzed by TRAP taining. (D) TRAP activity assay. Data are presented as the mean±SD of three independent experiments.The data represent three experiment exhibiting similar results. * p<.05 versus si-con+inhibitor-con(+RANKL); # p<.05 versus si-CXCR2+inhibitor(+RANKL).RANKL,receptor activator of nuclear factor-kB ligand; TRAP,tartrate-resistant acid phosphatase.

**Fig.6. The si-CXCR2 weakened the upregulatory effect of miR-155-5p inhibitor on the expression of osteoclastic bone resorption-related enzymes in RAW264.7 cells.**① si-con+inhibitor-con; ② si-con+inhibitor; ③si-CXCR2+inhibitor-con; ④

si-CXCR2+inhibitor. (A)The bone resorption-related enzyme mRNA expression levels were detected by RT-PCR. (B) The bone resorption-related enzyme protein expression levels were detected by western blotting.Data are presented as the mean±SD of three independent experiments.The data represent three experiment exhibiting similar results. * $p<.05$ versus si-con+inhibitor-con(+RANKL); # $p<.05$ versus si-CXCR2+inhibitor(+RANKL). RANKL,receptor activator of nuclear factor-kB ligand.

**Tables**

**Table 1.The sequences of mimic-con/mimic,inhibitor-con/inhibit,and si-con/si-CXCR2.**

| Name | F/R | sequences |
|---|---|---|
| mimic-con | -- | 5’-UCUCCGAACGUGU CACGUTT-3’ |
| mimic | -- | 5’-UGGGGAUAGUGUUAAUCGUAAUU-3’ |
| inhibitor-co n | -- | 5’ CAGUACUUUUGUGUAG UACAA-3’ |
| inhibitor | -- | 5’-CCCCUAUCACGAUU AGCAUUAA-3’ |
| si-con | Forwards | CTGGTCATCTTATACAGCATTCAAGAGATGCTG TATAAGATGACCAGTTTTT |
| | Reverse | TAAAACTGGTCATCTTATACAGCATCTCTTGAA TGCTGTATAAGATGACCAGGGG |
| si-CXCR2 | Forwards | CCCCTTCTCCGAACAGTGTCACGTTTCAAGAG AACGTGACACGTTCGGAGAATTTTT |
| | Reverse | TAAAAATTCTCCGAACGTGTCACGTTCTCTTG AAACGTGACACGTTCGGAGAAGGG |

**Fig. 1**

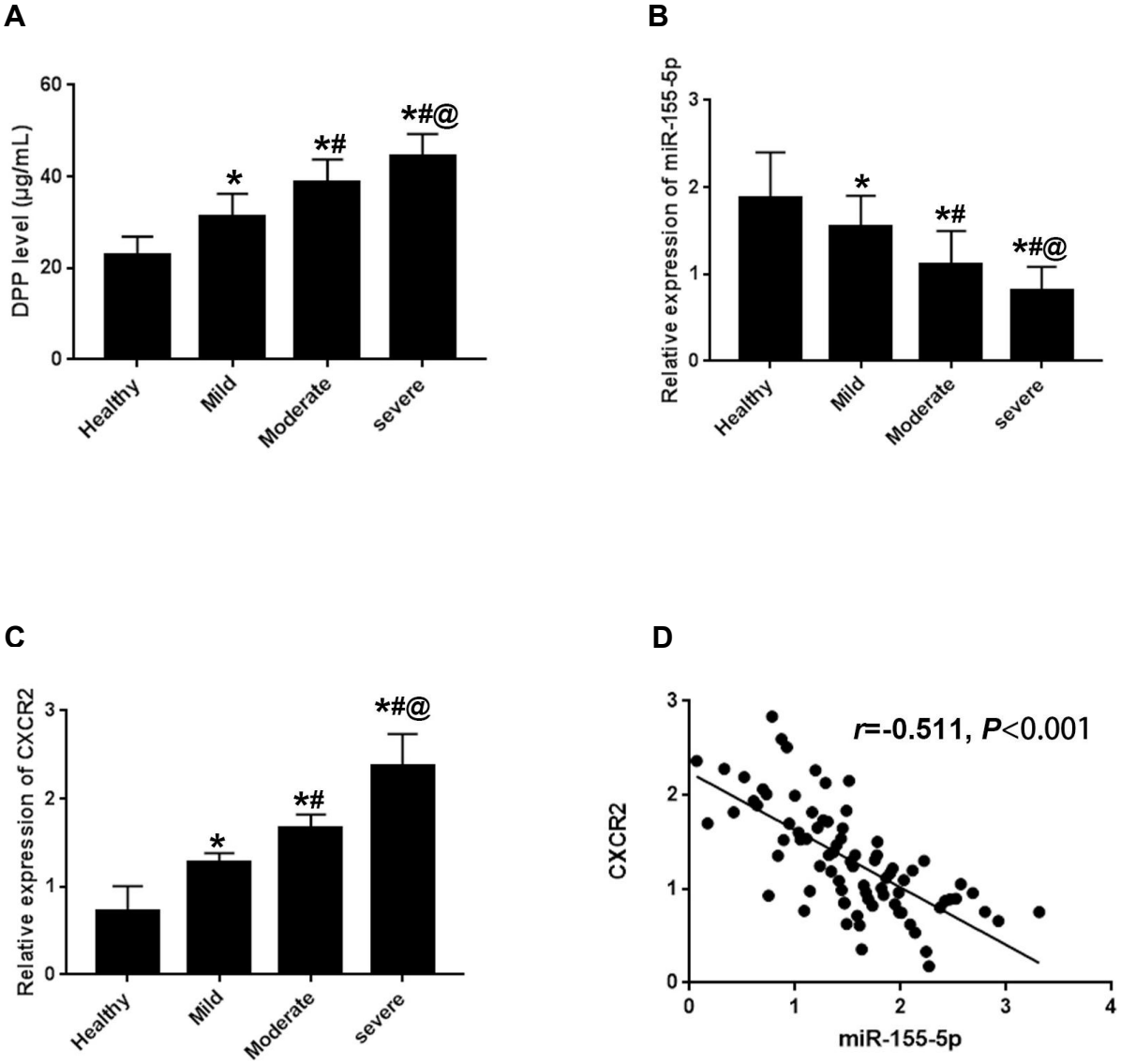
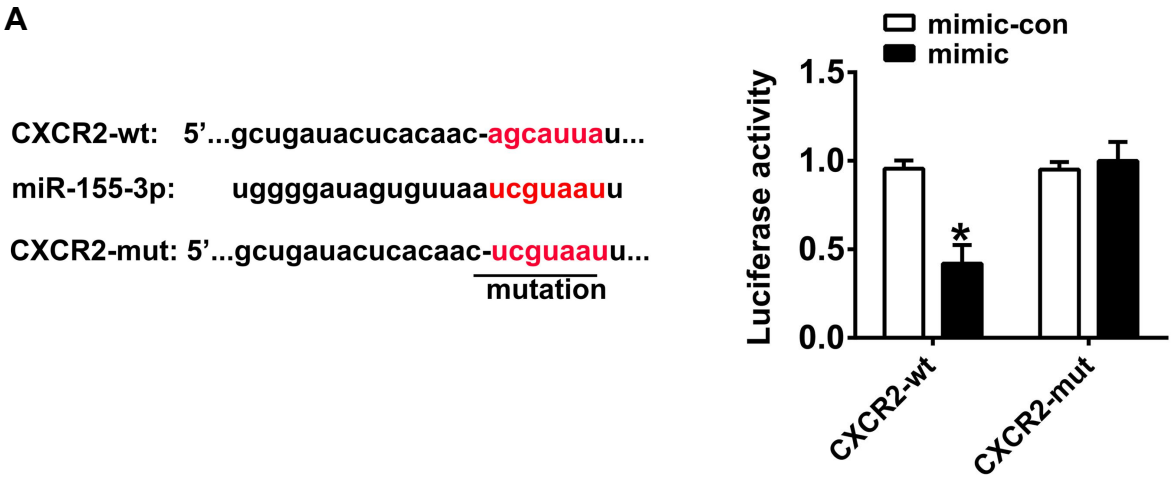
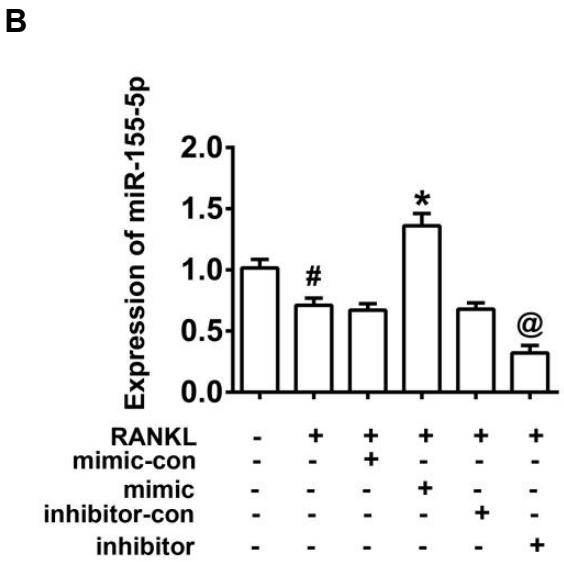
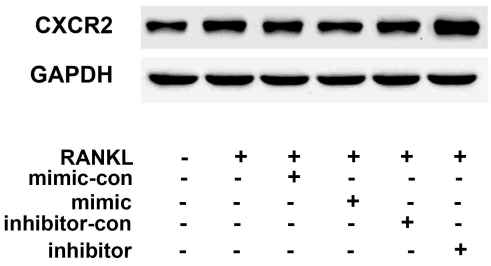

A
DPP level (μg/mL)
60
40
20
0
*
*#
*#@
Healthy
Mild
Moderate
severe
B
Relative expression of miR-155-5p
3
2
1
0
*
*#
*#@
Healthy
Mild
Moderate
severe
C
Relative expression of CXCR2
3
2
1
0
*
*#
*#@
Healthy
Mild
Moderate
severe
D
CXCR2
r=-0.511, P<0.001
3
2
1
0
0
1
2
3
4
miR-155-5p

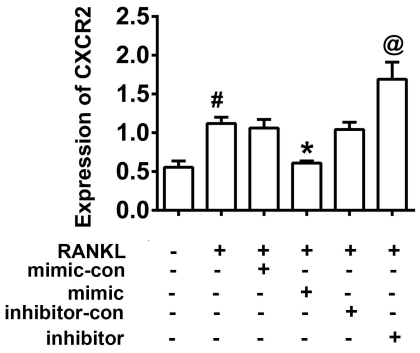
Fig. 2

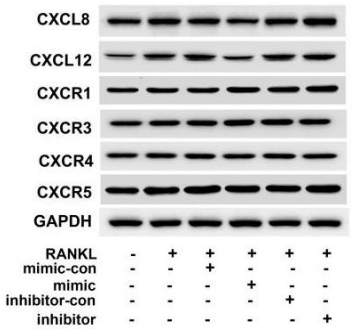

A
CXCR2-wt: 5'...gcugauacucacaac-agcauuau...
miR-155-3p: uggggauaguguuaaucguaauu
CXCR2-mut: 5'...gcugauacucacaac-ucguaauu...
mutation
mimic-con
mimic
Luciferase activity
1.5
1.0
0.5
0.0
*
CXCR2-wt
CXCR2-mut

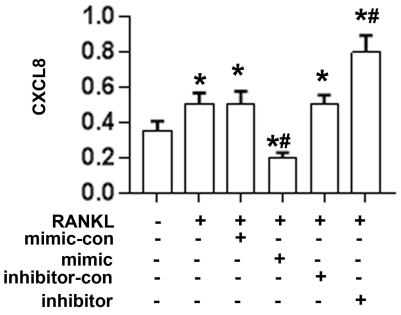

B
Expression of miR-155-5p
2.0
1.5
1.0
0.5
0.0
#
*
@
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +

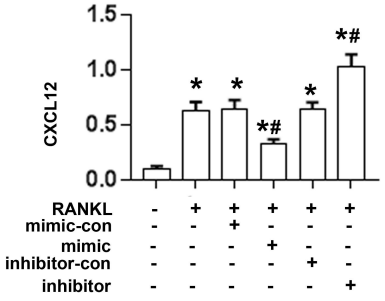
C


CXCR2
GAPDH
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +


Expression of CXCR2
2.5
2.0
1.5
1.0
0.5
0.0
#
*
@
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +


CXCL8
CXCL12
CXCR1
CXCR3
CXCR4
CXCR5
GAPDH
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +


CXCL8
1.0
0.8
0.6
0.4
0.2
0.0
*
*#
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +


CXCL12
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1.0
0.5
0.0
*
*#
RANKL - + + + + +
mimic-con - - + - - -
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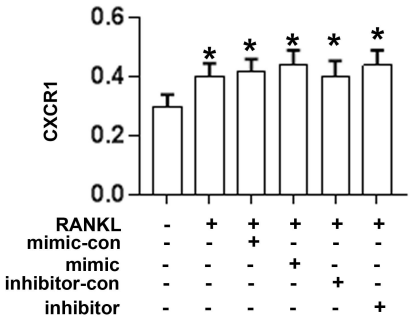
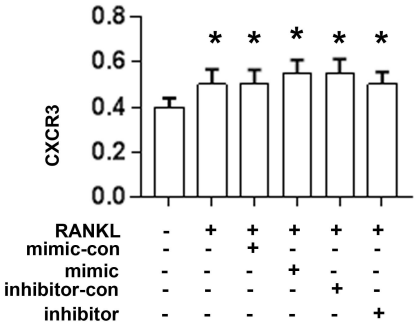
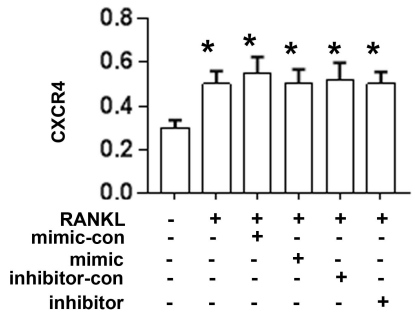
inhibitor-con - - - - + -
inhibitor - - - - - +


CXCR1
0.6
0.4
0.2
0.0
*
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +


CXCR3
0.8
0.6
0.4
0.2
0.0
*
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +


CXCR4
0.8
0.6
0.4
0.2
0.0
*
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +

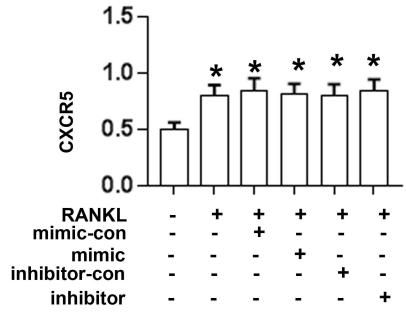

CXCR5
1.5
1.0
0.5
0.0
*
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +

Fig. 3

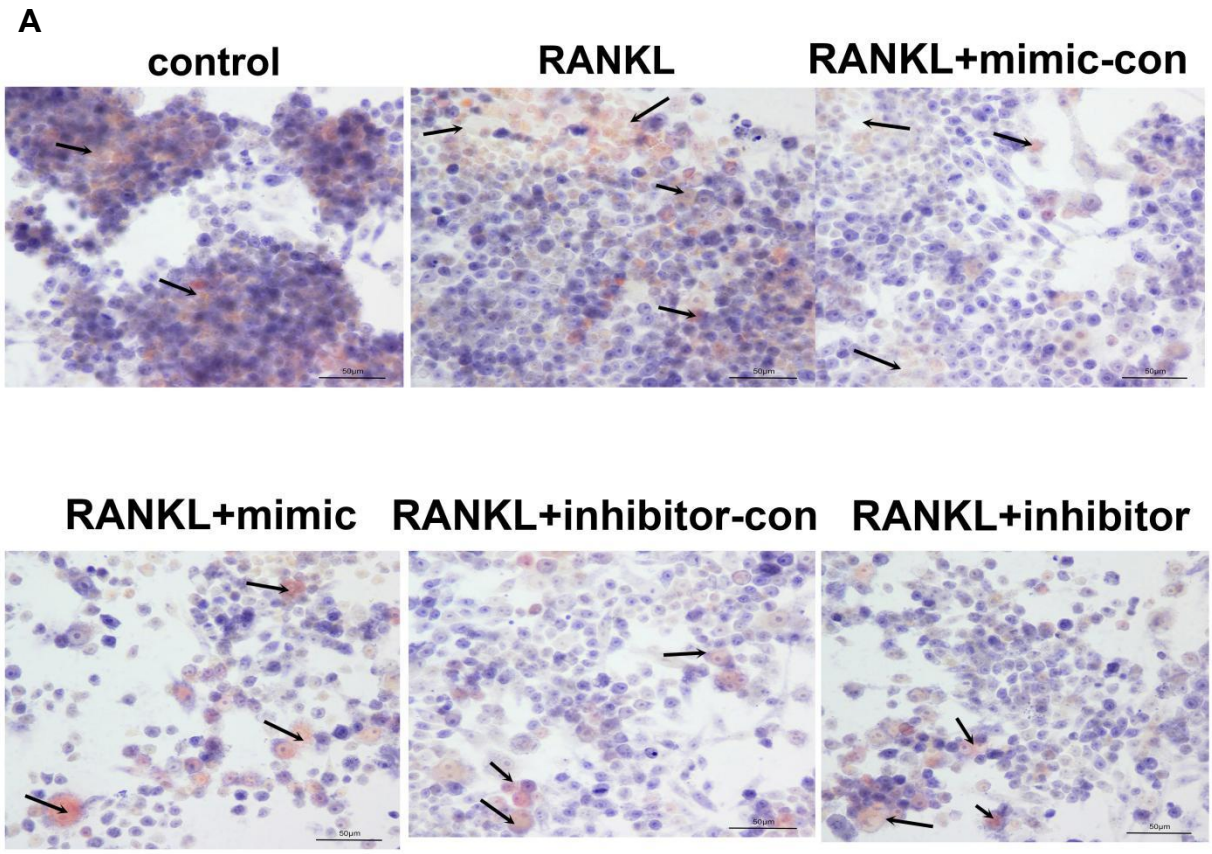

A
control
RANKL
RANKL+mimic-con
RANKL+mimic
RANKL+inhibitor-con
RANKL+inhibitor
B
Number of cells
400
300
200
100
0
#
*
@
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +
C
TRAP activity (% of control)
300
200
100
0
#
*
@
RANKL - + + + + +
mimic-con - - + - - -
mimic - - - + - -
inhibitor-con - - - - + -
inhibitor - - - - - +

**Fig. 4**

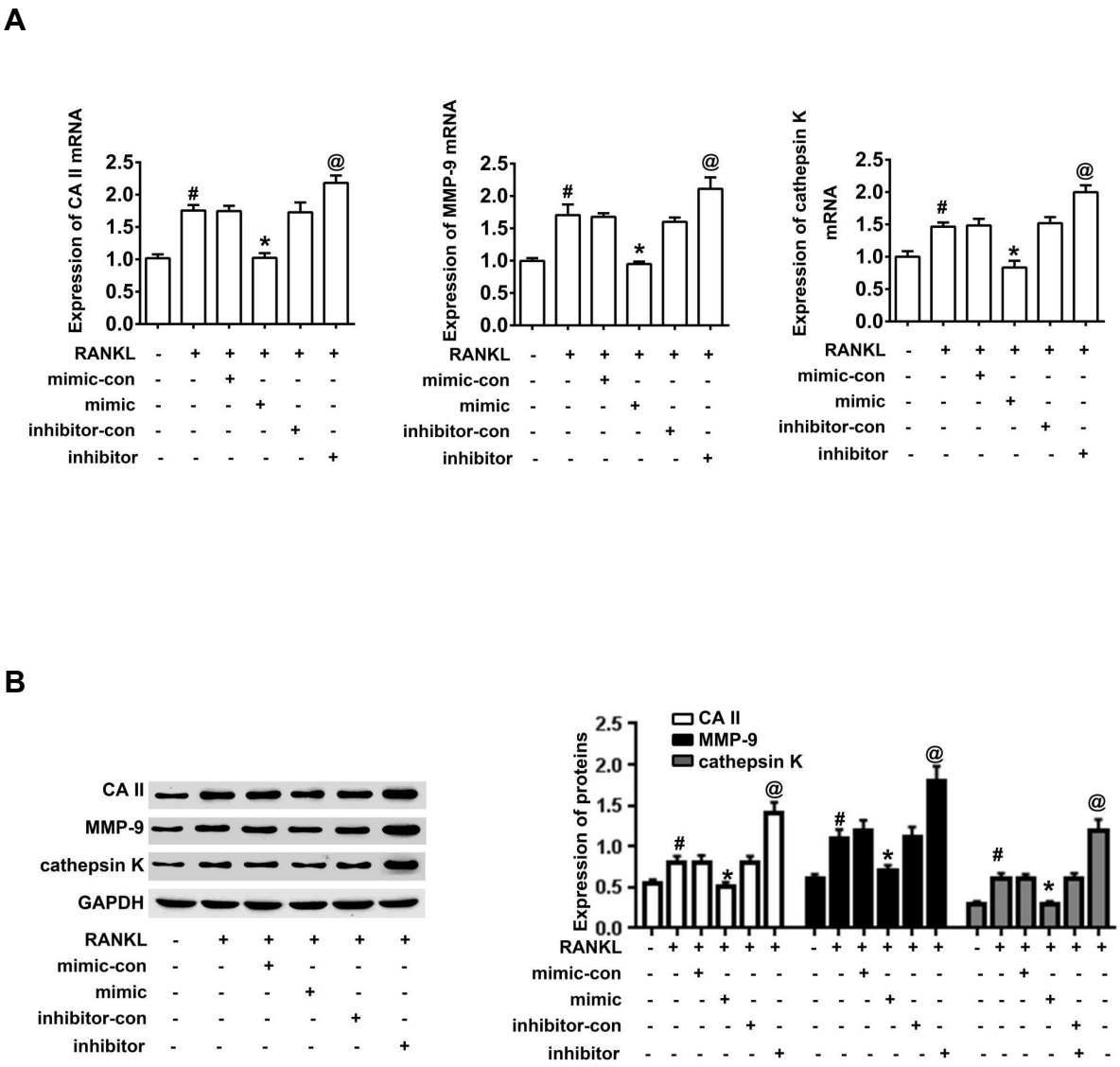
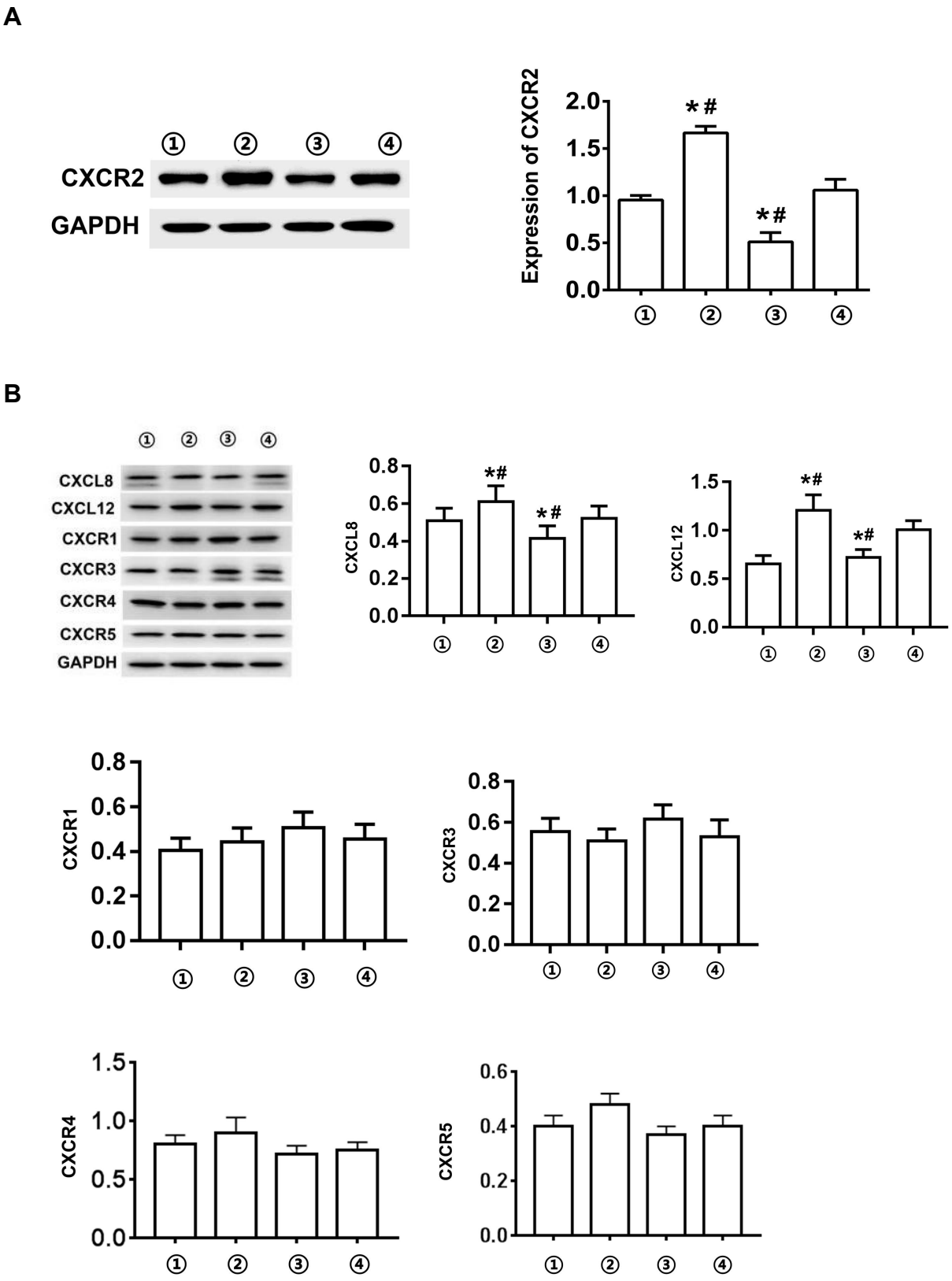
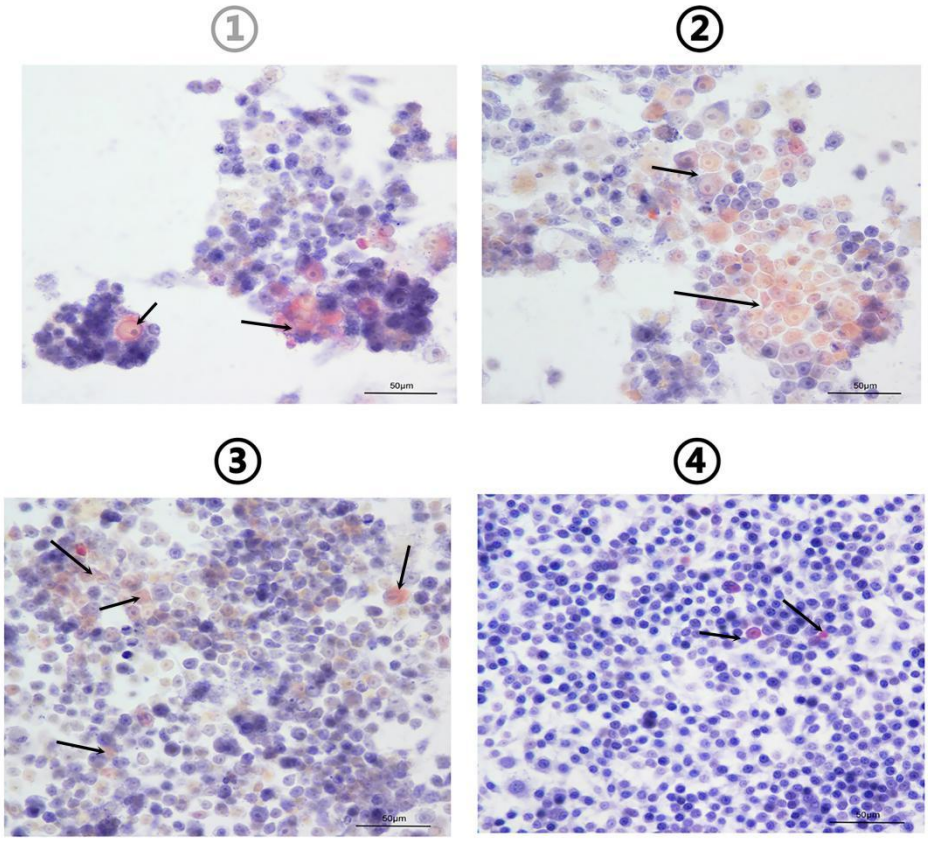

A
Expression of CA II mRNA
Expression of MMP-9 mRNA
Expression of cathepsin K mRNA
RANKL
mimic-con
mimic
inhibitor-con
inhibitor
B
CA II
MMP-9
cathepsin K
GAPDH
Expression of proteins

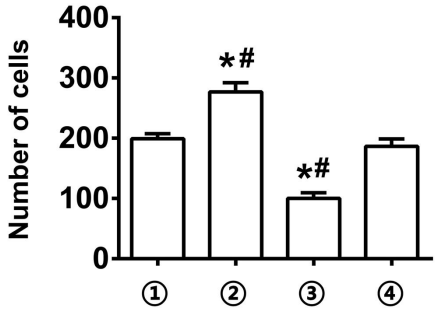
Fig. 5


A
①
②
③
④
CXCR2
GAPDH
Expression of CXCR2
2.0
1.5
1.0
0.5
0.0
*#
*#
B
CXCL8
CXCL12
CXCR1
CXCR3
CXCR4
CXCR5
GAPDH
0.8
0.6
0.4
0.2
0.0
1.5
1.0
0.5
0.6

C


①
②
③
④
50μm

D


Number of cells
400
300
200
100
0
*#
*#
①
②
③
④

E

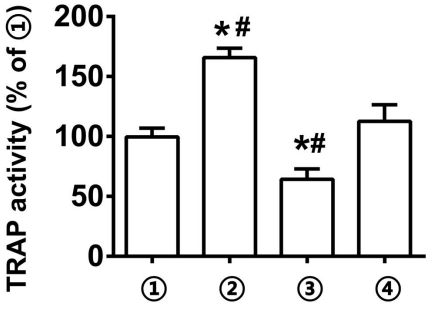
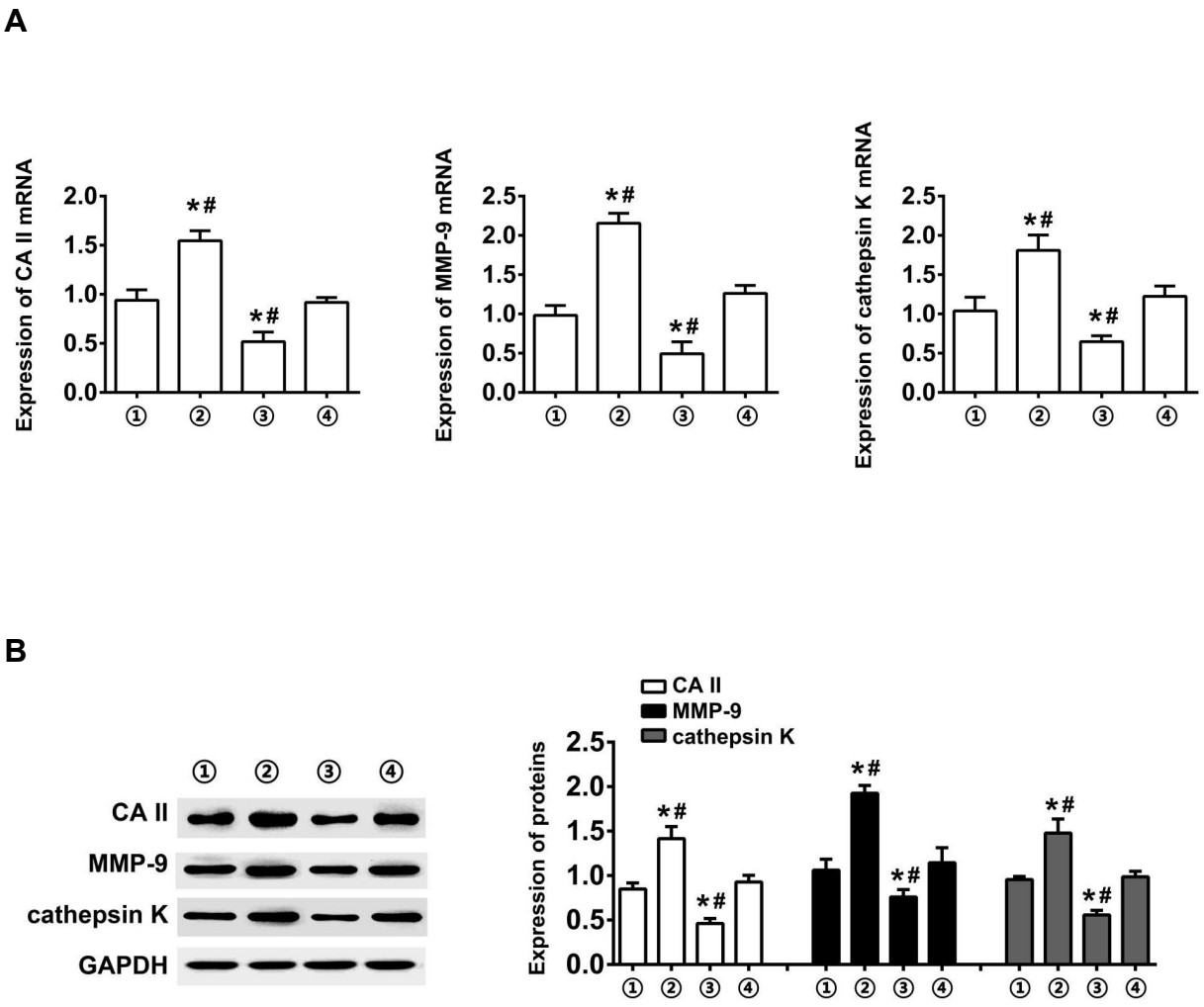

TRAP activity (% of ①)
200
150
100
50
0
*#
*#
①
②
③
④

Fig. 6


A
Expression of CA II mRNA
Expression of MMP-9 mRNA
Expression of cathepsin K mRNA
*#
①
②
③
④
B
CA II
MMP-9
cathepsin K
GAPDH
Expression of proteins