RHEUMATOLOGY

Original article

doi:10.1093/rheumatology/kex233

Downregulation of miR-106b attenuates inflammatory responses and joint damage in collagen-induced arthritis

Yunxia Tao[1,*], Zhirong Wang[2,*], Liangliang Wang[1,*], Jiawei Shi[1], Xiaobin Guo[1], Wei Zhou[1], Xiexing Wu[1], Yu Liu[1], Wen Zhang[3], Huilin Yang[1], Qin Shi[1], Yaozeng Xu[1] and Dechun Geng[1]

Abstract

Objective. miRNAs are small, signal-strand, non-coding RNAs that function in post-transcriptional regulation. We analysed the *in vivo* effect of miR-106b (miR-106b-5p) on inflammatory bone loss in CIA mice.

Methods. CIA mice are developed by injecting DAB/1 mice with bovine type II collagen containing Freund's adjuvant and then the *in vivo* effect of miR-106b is examined. On day 22, mice were given lentiviral negative control, lentiviral-mediated miR-106b mimics or lentiviral-mediated miR-106b inhibitor via orbital injection on a weekly basis. Morphological changes in the ankle joints were assessed via micro-CT and histopathology and cytokine expression levels were examined via immunohistochemical staining, ELISA or flow cytometric analysis. miR-106b and osteoclastic-related gene expression was evaluated via quantitative real-time PCR.

Results. CIA mice were found to have increased miR-106b expression and CIA-associated bone loss and inflammatory infiltration. miR-106b inhibitor treatment markedly decreased arthritis incidence and attenuated bone destruction and histological severity compared with the control group. Moreover, miR-106b inhibitor treatment suppressed RANK ligand (RANKL) expression, increased osteoprotegerin (OPG) expression and reduced the RANKL:OPG ratio in CIA mice. miR-106b inhibition also significantly decreased inflammatory mediator production in joint sections and reduced serum pro-inflammatory cytokine levels when compared with the control group. Additionally, miR-106b inhibition decreased tartrate-resistant acid phosphatase–positive cell numbers and suppressed murine bone marrow macrophage differentiation.

Conclusion. These findings indicate that miR-106b inhibition can ameliorate CIA-associated inflammation and bone destruction and thus may serve as a potential therapeutic for human RA treatment.

Key words: rheumatoid arthritis, miR-106b, collagen-induced arthritis, joint destruction, inflammation

BASIC SCIENCE

Rheumatology key messages

- miR-106b upregulation in CIA joints was associated with extensive bone loss and inflammatory synovitis.
- miR-106b inhibition reduced arthritis severity and prevented inflammatory bone destruction in CIA mice.
- miR-106b may be a promising candidate for treating RA and inflammation-related bone disease.

[1]Department of Orthopedics, First Affiliated Hospital of Soochow University, Suzhou, [2]Department of Orthopedics, Zhangjiagang Hospital of Traditional Chinese Medicine, Jiangsu and [3]Orthopedic Institute, Soochow University, Suzhou, China

Submitted 22 December 2016; revised version accepted 23 May 2017

*Yunxia Tao, Zhirong Wang and Liangliang Wang contributed equally to this study.

Correspondence to: Dechun Geng, Department of Orthopedics, First Affiliated Hospital of Soochow University, 188, Shi Zi Road, Suzhou, 215006, China.
E-mail: szgengdc@163.com

Introduction

RA, an autoimmune disorder, is characterized by synovial hyperplasia, chronic inflammation and the secretion of autoantibodies and many pro-inflammatory cytokines, which together cause joint destruction [1]. Chronic inflammation-stimulated bone destruction is a critical pathological feature in RA and is mediated by osteoclastic bone resorption [2, 3]. Osteoclasts, which are specialized bone-resorbing cells, are derived from monocyte/macrophage differentiation in the

presence of two essential osteoclastogenic factors: RANK ligand (RANKL) and M-CSF [4]. The pathological microenvironment of the arthritic synovium causes excessive osteoclast differentiation and activation, resulting in extensive bone loss. Additionally, chronic inflammation also disrupts the balance of bone remodelling by directing it towards bone resorption rather than bone formation [5]. This imbalance results in systemic osteopenia and a high risk of osteoporotic fractures [6, 7]. Thus, better RA treatments focused on quenching inflammatory signalling and halting bone damage are needed.

miRNAs are small, signal-strand, non-coding RNAs (~19–23 nucleotides) that function in post-transcriptional regulation in diverse biological processes such as proliferation, differentiation and cell apoptosis under both physiological and pathological conditions. In mice, the overexpression of miR-23b has been shown to protect against IL-17-induced autoimmune inflammation [8]. Moreover, miR-140 knockout mice exhibit defective cartilage and skeletal formation [9] and miR-124 inhibits osteoclast differentiation and suppresses adjuvant-induced arthritis *in vivo* [10]. Meanwhile, abnormal expression of miR-155 and miR-146a was observed in inflamed RA joints and was closely associated with the upregulation of pro-inflammation cytokines [11]. Additionally, miR-155$^{-/-}$ mice or mice treated with miR-146a are resistant to CIA, with a significant suppression of inflammatory synovial hyperplasia and markedly reduced joint destruction also noted [12, 13]. Thus it is not surprising that miRNAs would be pursued as a suitable method for the treatment of RA-induced bone destruction.

miR-106b, a well-characterized oncogenic miRNA, belongs to the miR-106b-25 cluster and is overexpressed in many human tumours such as gliomas, stomach, prostate and kidney [14]. Recently, several authors have demonstrated that miR-106b is a key player in the processes of inflammation, hypersensitivity and some autoimmune disease [15–18]. Interestingly, one study showed that miR-106b is a suitable target for the treatment of giant cell tumour–induced bone resorption [19], while another study demonstrated that miR-106b directly regulates MMP2 expression and participates in the process of breast cancer bone metastasis [20]. Furthermore, miR-20a, a homologue to miR-106b, reportedly regulates pro-inflammatory cytokine expression during RA [21]. However, until now, no comprehensive study examining the effects of miR-106b in RA-induced inflammatory processes or bone destruction had been performed. We hypothesized that miR-106b modulates inflammatory bone destruction in the RA process.

This study was undertaken to investigate whether RA associated chronic inflammation and joint destruction are related to miR-106b production, and whether regulates the expression of miR-106b could affect inflammatory bone loss in CIA mice.

Methods

Antibodies and reagents

Bovine type II collagen, Freund's adjuvant and a tartrate-resistant acid phosphatase (TRAP) kit were obtained from Sigma (St Louis, MO, USA). Recombinant mouse RANKL and M-CSF were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against RANKL, osteoprotegerin (OPG), TNF-α, IL-1β, IL-6 and cyclooxygenase-2 (COX-2) were obtained from Abcam (Shanghai, China). Murine ELISA kits for RANKL and OPG were obtained from R&D Systems. Murine TNF-α, IL-1β and IL-6 Flex Sets were from Univ-bio (Shanghai, China). A lentiviral negative control (NC), miR-106 mimics and miR-106b inhibitor were purchased from Gene Pharma (Shanghai, China).

Induction of CIA

All experiments involving animal use were approved by the Institutional Animal Ethics Committee of the First Affiliated Hospital of Soochow University. Male DBA/1 mice (8–10 weeks old) were obtained from the Animal Center of Soochow University and were housed under specific pathogen-free conditions. Bovine type II collagen was reconstituted in 10 mM acetic acid at 4 °C for 12 h and emulsified with the same volume of Freund's complete or incomplete adjuvant. Experimental arthritis was induced in DBA/1 mice via intradermal injection with 100 μl of emulsion containing 200 μg of bovine type II collagen at the base of the tail. At 21 days after the primary immunization, a booster consisting of 200 μg bovine type II collagen emulsified in an equal volume of Freund's incomplete adjuvant was delivered near the first injection site. On day 22, the mice were given 0.2 ml lentiviral NC ($n = 10$), lentiviral-mediated miR-106b mimics ($n = 10$) or lentiviral-mediated miR-106b inhibitor ($n = 10$) containing 1×10^8 U/ml via orbital injection on a weekly basis. In the sham group, an equal volume of PBS was administered in the same manner. Paw swelling was observed by two independent investigators with a Vernier calliper. Arthritis was evaluated and scored from 0 to 4 according to the following method: 0, no signs of arthritis; 1, swelling and/or redness of the paw or one digit; 2, two joints involved; 3, more than two joints involved; 4, severe arthritis of the entire paw and all digits [22, 23]. Serum samples were obtained just prior to sacrifice and the hind paws were collected and prepared for molecular, histological and radiologic analyses.

Micro-CT analysis

Dissected paws and L2 lumbar vertebrae were scanned as described previously for mouse bone [22, 24] and following the guidelines for assessment of bone microstructure in rodents using micro-CT (SkyScan 1176, SkyScan, Aartselaar, Belgium) [25]. Briefly, scanning was done at a voxel size of 9 μm, X-ray tube voltage of 70 kV, current of 141 μA and exposure time of 1750 ms. The X-ray projections were obtained at 0.5° intervals with a scanning angular rotation of 360°. CT Analyzer software was used for morphometric quantification of trabecular bone parameters, including bone volume fraction (BV/TV; %) and trabecular thickness (TbTh; mm).

Histological and immunohistochemical analysis

For histopathological analysis, the knee joints were fixed, decalcified and paraffin embedded as previously described [13]. Five-micrometre sections were prepared and stained with haematoxylin and eosin (H&E) or safranin O/fast green. The stained sections were then scored on a scale of 0–4 [26] for changes in cartilage erosion, inflammatory response and bone damage. To investigate osteoclast bone resorption activity, sections were stained with a TRAP staining kit according to the manufacturer's protocols. TRAP-positive multinucleated cells located at the bone surface within the bone destruction area were considered to be mature osteoclasts and were counted microscopically.

For immunohistochemical analysis, sections were incubated with primary antibodies against murine RANKL, OPG, TNF-α, IL-1β, IL-6 or COX-2 followed by the appropriate secondary antibodies. Rinsed sections were counterstained with haematoxylin. After immunohistochemical staining, four consecutive slices were scored semi-quantitatively for each cytokine by two pathologists. A score of 0 represented minimal expression, 1 represented mile expression, 2 represented moderated expression and 3 represented abundant expression [26].

Measurement of serum pro-inflammatory cytokines, RANKL and OPG

Serum TNF-α, IL-1β and IL-6 levels in CIA mice were determined by flow cytometric analysis using a commercial kit according to the manufacturer's instructions. Serum RANKL and OPG levels were determined using specific ELISA kits.

Osteoclast culture and TRAP staining

Bone marrow cells were collected and cultured for 16 h with 30 ng/ml M-CSF. Non-adherent cells were harvested, while red blood cells were lysed and incubated with M-CSF (30 ng/ml) and RANKL (50 ng/ml) for 5 days. Mature osteoclasts were visualized via TRAP staining, with TRAP-positive cells with three or more nuclei considered osteoclasts.

Quantitative real-time PCR

To quantify mRNA expression, total RNA was isolated and reverse transcribed to cDNA using reverse transcriptase (Takara, Otsu, Japan). Quantitative real-time PCR was performed using a Takara TP800 PCR Thermal Cycler Dice Detection System (Takara), with glyceraldehyde 3-phosphate dehydrogenase used as an internal control. All the mouse primer sequences were as follows: TRAP forward 5′-CTGGAGTGCACGATGCCAGCGACA-3′ and reverse 5′- TCCGTGCTCGGCGATGGACCAGA-3′; nuclear factor of activated T cells 1 forward 5′- CCGTTGC TTCCAGAAAATAACA-3′ and reverse 5′-TGTGGGATGTG AACTCGGAA-3′; cathepsin K (Cath-K) forward 5′-CTTCC AATACGTGCAGCAGA-3′ and reverse 5′- TCTTCAGGGC TTTCTCGTTC-3′; MMP-9 forward 5′- CAAAGACCTGAAA ACCTCCAA-3′ and reverse 5′-GGTACAAGTATGCCTCTG CCA-3′; glyceraldehyde 3-phosphate dehydrogenase forward 5′-ACCCAGAAGACTGTGGATGG-3′ and reverse 5′- CACATTGGGGGTAGGAACAC-3′.

Total miRNA was isolated using a miRNA Isolation kit (Biochain Institute, Newark, CA, USA) according to the manufacturer's instructions. Aliquots of total miRNA were subjected to reverse transcription and PCR amplification using a TP800 PCR Thermal Cycler Dice Detection System (Takara). Specific primers for miR-106b and U6 were obtained from Ambion (Foster City, CA, USA).

Statistical analysis

All values are expressed as a mean (S.E.M.). Differences between groups were analysed by a one-way analysis of variance for multiple comparisons where appropriate. *P*-values <0.05 were considered significant. SPSS 11.0 software (SPSS, Chicago, IL, USA) was used to carry out the statistical computations.

Results

miR-106b ameliorates disease severity in CIA mice

To begin, endogenous miR-106b expression was examined in inflamed mice joints, with higher levels observed in CIA mice compared with non-CIA mice (Fig. 1A). Considering the important role of miR-106b in bone remodelling, a lentiviral-based gene transfer system was developed to verify whether modulating miR-106b signalling affects disease progression in CIA mice. As expected, mature miR-106b was significantly decreased in the joints of CIA mice injected with miR-106b inhibitor relative to controls (Fig. 1A). Meanwhile, miR-106b inhibitor treatment significantly suppressed arthritis development in CIA mice, with significantly lower mean arthritis scores and reduced paw thicknesses noted relative to the control group (Fig. 1B–D).

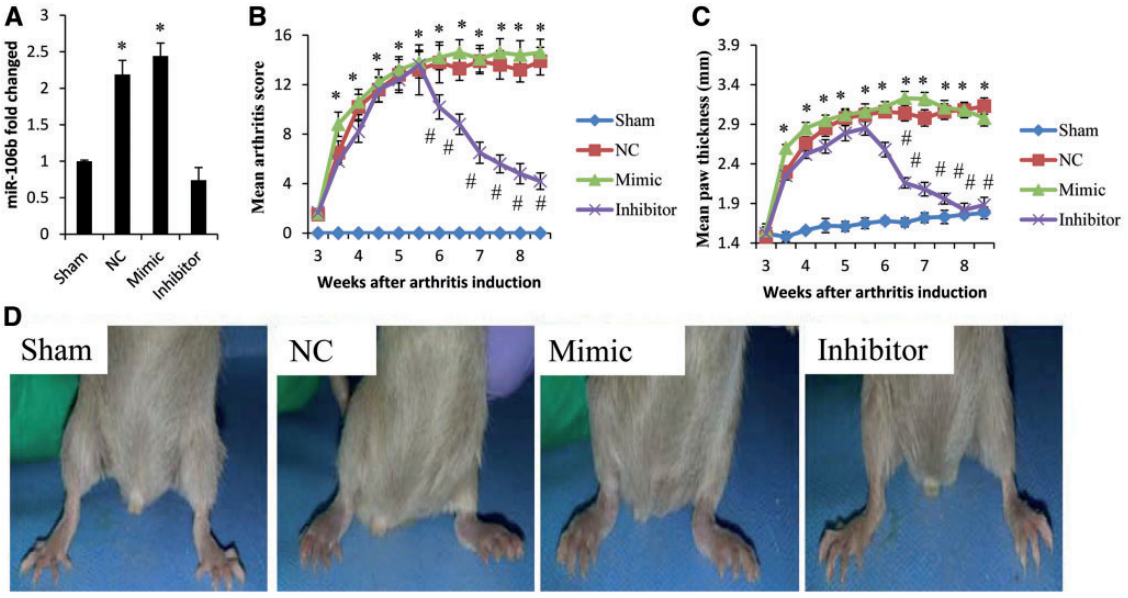
miR-106b improved the morphology of arthritic joints

When examining CIA mice knee joints, histological analysis revealed significant inflammatory cell infiltration, synovial hyperplasia, cartilage damage and bone erosion (Fig. 2A and B). In contrast, CIA mice treated with miR-106b inhibitor showed a significant reduction in inflammatory cell infiltration, synovial hyperplasia, cartilage damage and bone erosion (Fig. 2C–E).

miR-106b impaired bone destruction in CIA mice

Next, micro-CT analysis was utilized to further investigate the protective effects of miR-106b inhibition in CIA mice. Mice treated with NC or miR-106b mimics showed extensive bone damage, while miR-106b inhibition greatly reduced bone destruction (Fig. 3A). Furthermore, micro-CT analysis of the inflamed joints also showed that the examined bone parameters, including BV/TV and TbTh, were significantly increased in mice treated with miR-106b inhibitor (Fig. 3B). We also performed micro-CT of L2 lumbar vertebrae to evaluate the effect of miR-106b inhibition on the skeletal system. Significant bone loss was observed in the NC and miR-106b mimics groups compared with the sham groups, and miR-106b inhibition

Fig. 1 miR-106b regulates CIA progression in mice



(**A**) Quantitative PCR analysis of miR-106b expression in ankle tissues from mice in different groups. Time-course analysis to determine (**B**) arthritis score and (**C**) paw thickness in non-CIA mice and CIA mice treated with lentiviral NC, lentiviral-mediated miR-106b mimics or lentiviral-mediated miR-106b inhibitor. (**D**) Hind paws of CIA mice on day 56. $^{*}P < 0.05$ compared with the sham group. $^{\#}P < 0.05$ compared with the NC group.

obviously suppressed bone destruction, indicated by increased BV/TV and TbTh (Fig. 3C and D). These findings further demonstrate that miR-106b inhibition can reduce bone destruction in CIA mice.

miR-106b modulates RANKL and OPG expression in CIA mice

To evaluate RANKL and OPG expression in inflamed CIA mice joints, immunohistochemical staining was utilized. In CIA knee joints, extensive RANKL expression was observed, with OPG expression only slightly upregulated. In contrast, miR-106b inhibitor–treated sections revealed a significant decrease in the numbers of RANKL-positive cells but showed a marked increase in OPG-positive cells when compared with NC-treated mice (Fig. 4A–C).

Serum from CIA mice was then examined via ELISA analysis and showed serum RANKL levels (43.67 ± 5.59 pg/ml) to be significantly increased when compared with the control group (16.17 ± 3.18 pg/ml; $P < 0.05$), whereas OPG levels (32.83 ± 6.84 ng/ml) were not significantly altered relative to the controls (36.08 ± 5.54 ng/ml; $P > 0.05$). In miR-106b inhibitor–treated mice, RANKL levels were significantly downregulated while OPG levels were upregulated. Consequently, the RANKL:OPG ratio increased from $(0.49 \pm 0.10)/10^3$ to $(1.36 \pm 0.39)/10^3$ in CIA mice and was reduced to $(0.48 \pm 0.08)/10^3$ in miR-106b inhibitor–treated mice (Fig. 4D–F).

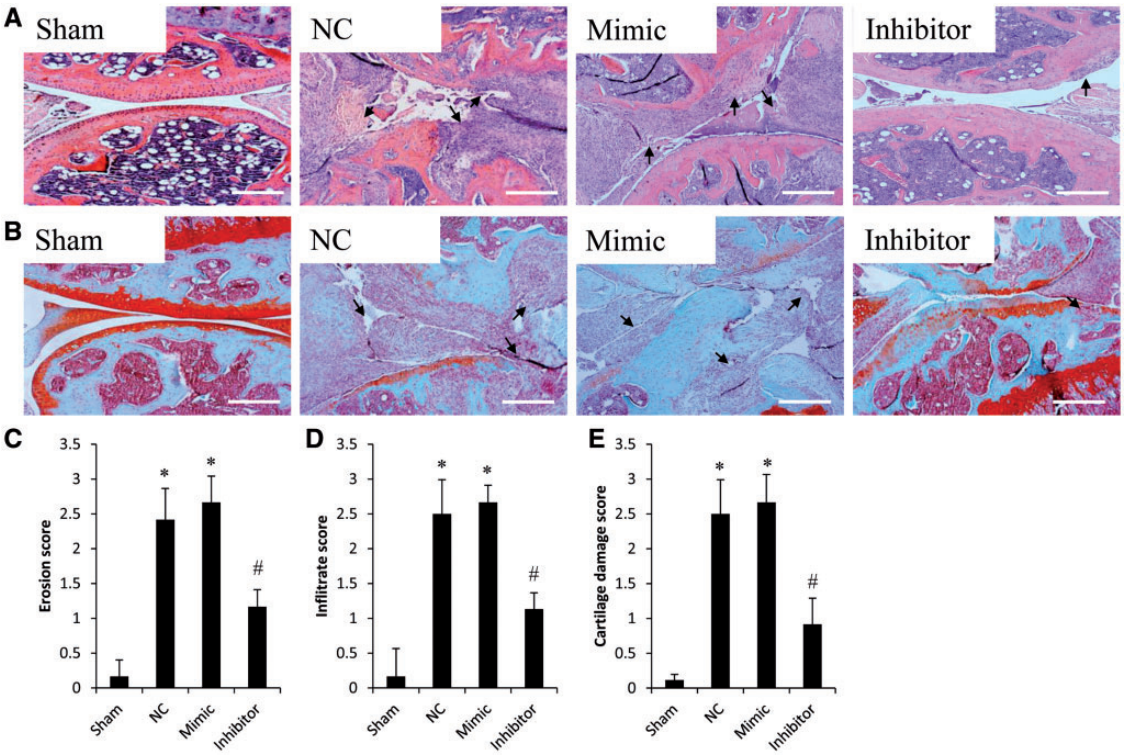
miR-106b inhibition reduces pro-inflammatory cytokine expression in CIA mice

Immunohistochemical staining of knee joint tissues collected from CIA mice stained positive for TNF-α, IL-6, IL-1β and COX-2, which were primarily localized in infiltrated cells around the joints. However, following miR-106b inhibitor treatment, few positive staining reactions were observed. (Fig. 5A). Semi-quantitative analysis showed an obvious decrease in TNF-α, IL-6, IL-1β, and COX-2 positive staining in miR-106b inhibitor–treated mice compared with the NC group. There was no TNF-α, IL-6, IL-1β, or COX-2 positive staining around joints obtained from the sham groups (Fig. 5B–E). To further substantiate these findings, serum TNF-α, IL-6 and IL-1β levels were analysed via ELISA. Consistent with the immunohistochemical staining results, miR-106b inhibition caused a significant reduction in serum TNF-α, IL-6 and IL-1β levels (Fig. 5F–H).

miR-106b inhibition decreases osteoclast numbers and suppresses osteoclast formation

To further characterize the role of miR-106b within the CIA bone microenvironment, its potential ability to reduced osteoclastic resorption was examined. Histological results showed that NC- and miR-106b mimic–treated groups had larger numbers of TRAP-positive cells located on eroded bone surfaces than in the vehicle-treated group. However, few TRAP-positive cells presented in the miR-106b inhibitor–treated group (Fig. 6A and C).

Fig. 2 Histopathological analysis of CIA mice treated with miR-106b



(**A**) Haematoxylin and eosin and safranin O/fast green staining of the ankle joints in CIA mice. Arrow indicated synovial effusion and mixed inflammatory cell infiltrates, or damaged cartilage (scar bar = 100μm). (**B**) Erosion score, (**C**) infiltrate score and (**D**) cartilage damage score determination. $^{*}P < 0.05$ compared with the sham group. $^{\#}P < 0.05$ compared with the NC group.

To examine whether miR-106b affects osteoclast formation, primary bone marrow macrophages were incubated with osteoclastogenic medium. As expected, miR-106b expression was significantly decreased in bone marrow macrophages treated with miR-106 inhibitor compared with the control groups (Fig. 6E). Moreover, TRAP-positive cell numbers were markedly decreased in the miR-106 inhibitor groups (Fig. 6B and D). Additionally, miR-106 inhibitor treatment significantly inhibited TRAP, nuclear factor of activated T cells 1, Cath-K and MMP9 mRNA levels, which are markers associated with osteoclast differentiation (Fig. 6F–I).

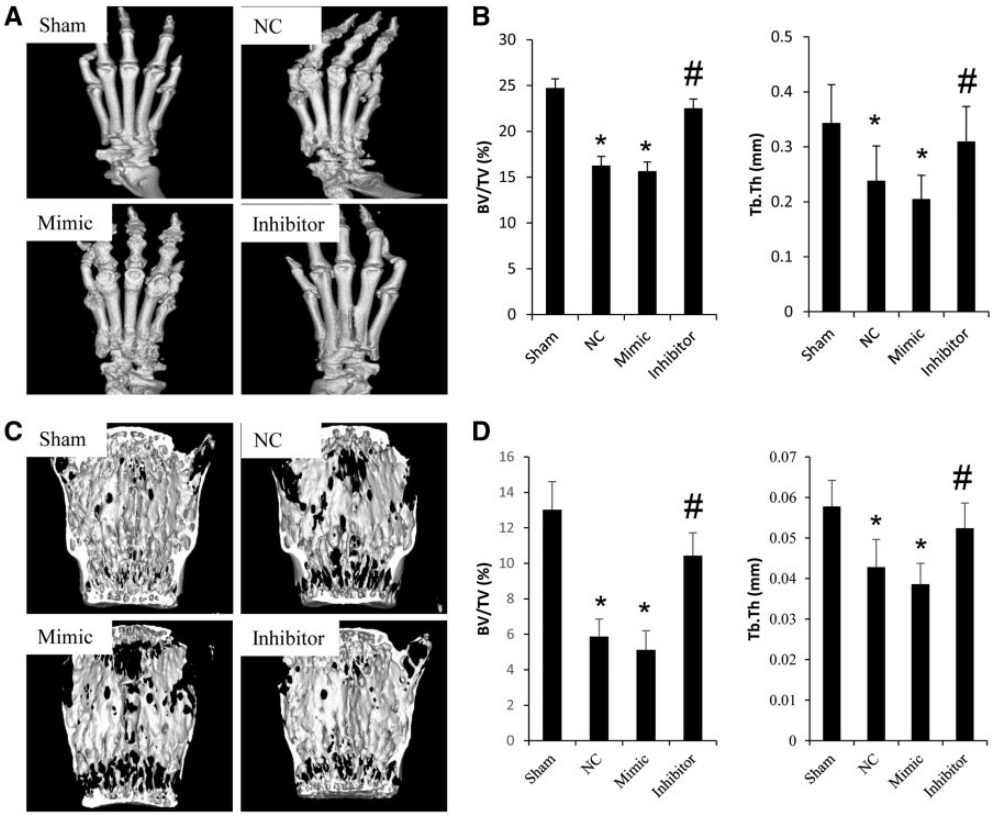
Discussion

RA is a complex autoimmune disease that is associated with inflammatory synovitis and bone and cartilage structural damage [1–3]. Several miRNAs have been shown to participate in RA progression [8–12]. However, unlike these other miRNAs, miR-106b has not been previously reported to have an important regulatory role in RA-induced inflammatory synovitis and bone loss. Herein, miR-106b upregulation was shown to be associated with extensive bone destruction in inflamed joints. However, the use of an inhibitor to suppress miR-106b expression improved joint swelling and reduced bone destruction in CIA mice, as confirmed by micro-CT and histopathological analyses. Thus these results suggest that miR-106b may be a promising candidate for treating RA-induced inflammatory joint destruction.

miR-106b, a member of the miR-106b-25 cluster, is located at chromosome 7 [27] and has been shown to be an oncogene and promote stem cell proliferation, migration and invasion in human cancers [14]. Recently, miR-106b has been shown to be critical in the regulation of bone remodelling [28] and bone resorption via the modulation of RANKL expression in an ovariectomized mouse model [19]. In the current study, miR-106b upregulation in inflamed CIA joints was shown to be associated with extensive bone loss and inflammatory synovitis. Thus the aim of this study was to further elucidate the role of miR-106b in CIA-associated bone loss, with hopes that these findings can be utilized to develop a therapeutic treatment for RA.

As expected, miR-106b downregulation significantly attenuates the severity of arthritis in CIA mice. In CIA mice, type II collagen inoculation caused a significant increase in the mean arthritis score and paw thickness, as well as promoting inflammatory cell infiltration, synovial hyperplasia, cartilage damage and bone erosion, which is

Fig. 3 miR-106b modulates bone destruction in CIA mice



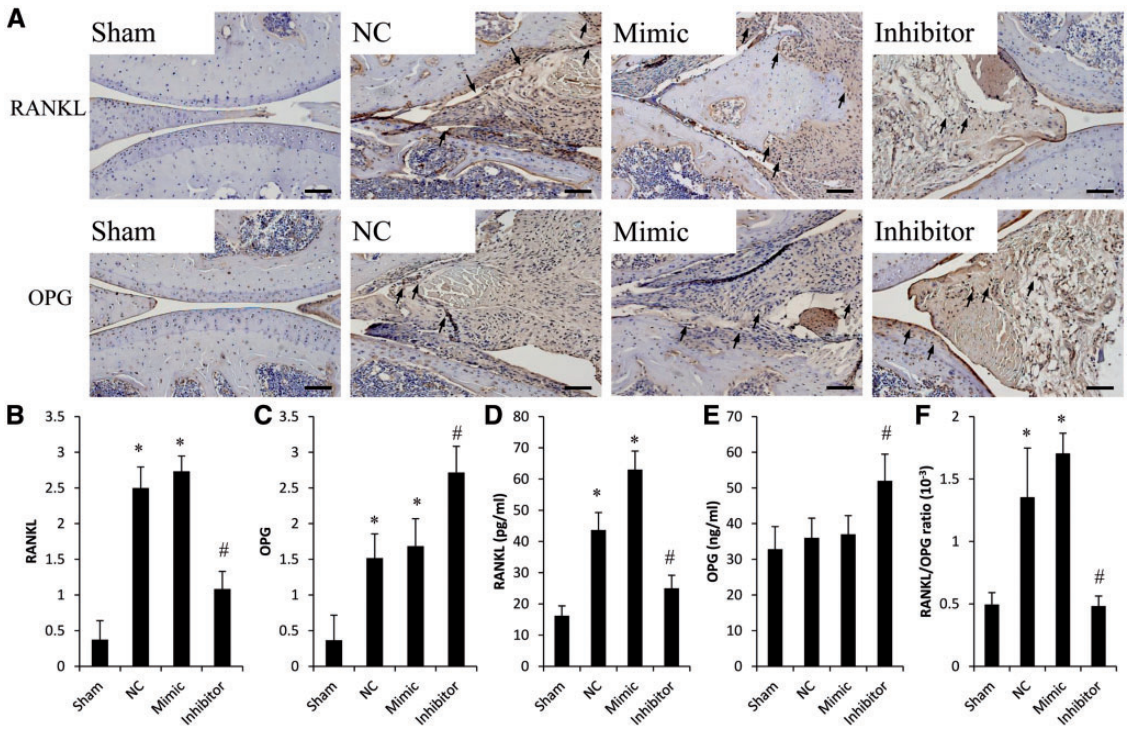
(**A**) Representative micro-CT images of the hind paws of CIA mice with different treatments. (**B**) BV/TV (%) and TbTh (mm) were determined. (**C** and **D**) Representative three-dimensional images and trabecular parameters of the L2 vertebrae are shown. $^{*}P < 0.05$ compared with the sham group. $^{\#}P < 0.05$ compared with the NC group.

consistent with previous studies [22, 26, 29]. In contrast, miR-106b inhibition in CIA mice significantly decreased CIA-induced inflammatory bone destruction.

In RA, the importance of osteoclast activation in the pathogenesis of focal bone loss has been verified in both patients and animal models [1, 26, 30]. Thus the inhibition of osteoclast differentiation and activation is a desirable approach to suppressing RA-associated bone loss [31]. In the current study, CIA mice had significantly increased numbers of TRAP-positive multinucleated cells in areas of pannus invasion into the inflamed joints. However, following miR-106b inhibition, TRAP-positive multinucleated cell numbers markedly decreased relative to the NC group. In a recent study, miR-106b downregulation was actually found to increase osteoclastogenesis and promoted bone resorption, thus acting via osteoclastogenic factor modulation [19]. However, this previous study focused on osteoclastogenesis in endocortical or periosteal bone, which could explain the observed expressional variation and suggests tissue-specific osteoclastogenesis in RA. Furthermore, the present study demonstrated that miR-106b inhibition suppresses osteoclast precursor differentiation and decreased osteoclast-associated gene expression. Moreover, miR-106b inhibitor treatment decreased osteoclast formation and differentiation, with TbTh and BV/TV also significantly decreased. These findings indicate that the protective effect exhibited by the miR-106b inhibitor is modulated through inhibition of osteoclastogenesis, thus decreasing RA-induced bone destruction.

RANKL promotes osteoclast differentiation and activation via RANK receptor binding on pre-osteoclasts and is an important player in bone destruction [4]. OPG, a decoy receptor of RANKL, disrupts the interaction between RANKL and RANK and thereby attenuates osteoclastic bone destruction [32]. Thus the RANKL:OPG ratio is critical in bone remodelling and regulates the activation stage of osteoclasts. Previous studies have demonstrated that during RA pathogenesis, RANKL expression is elevated while OPG expression is suppressed, thus further activating osteoclasts and promoting bone destruction. This finding indicates that the RANKL:OPG ratio is critical for RA-induced bone destruction [32]. Additionally, other studies have demonstrated that downregulated RANKL or upregulated OPG expression can effectively attenuate RA-associated focal bone loss [33, 34]. In the current study, immunohistochemical staining showed that CIA mice treated with miR-106b inhibitor had increased OPG

FIG. 4 Expression of RANKL and OPG in CIA mice



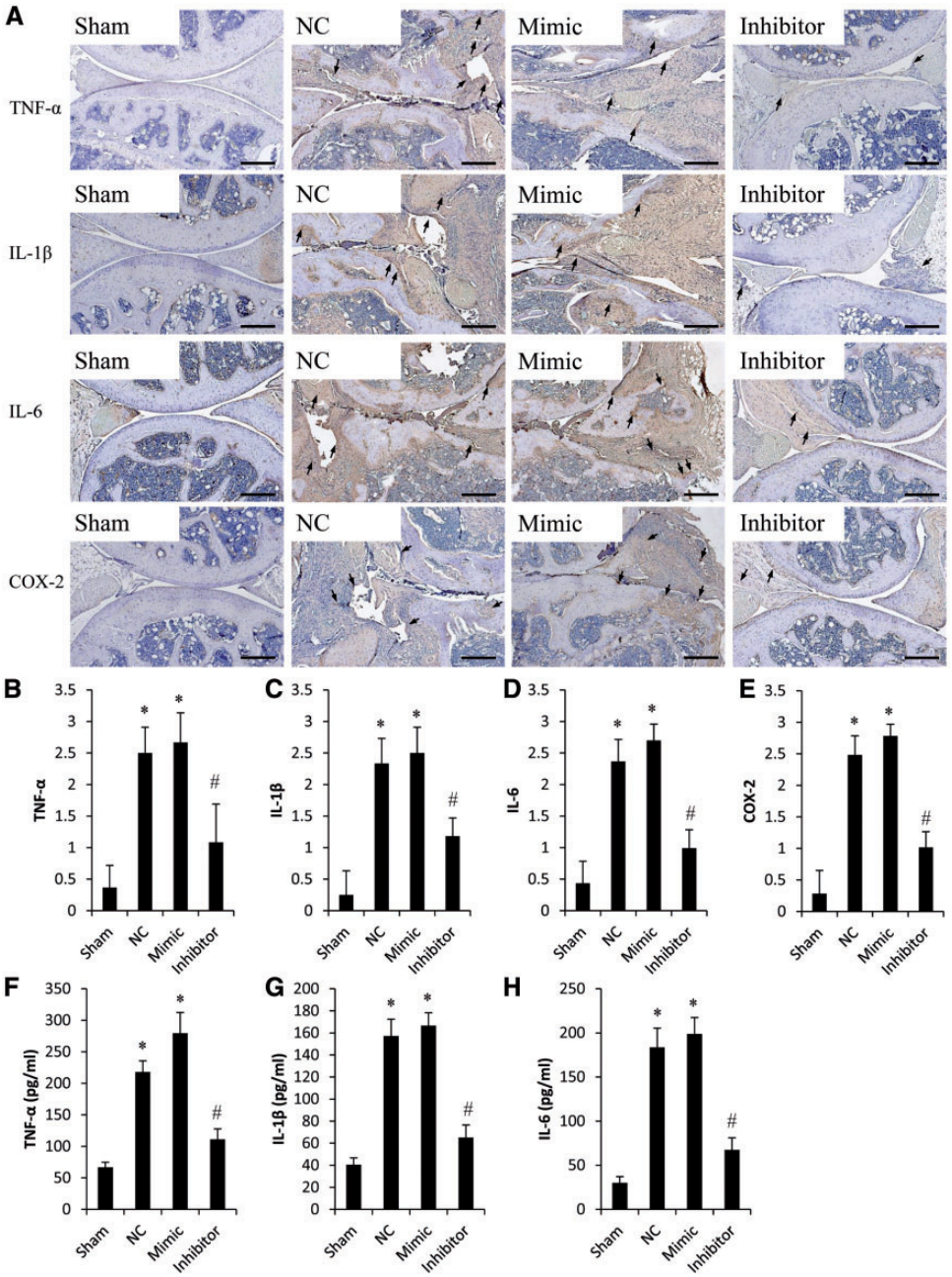
(**A**) Representative immunohistochemical images of RANKL and OPG (brown, indicated by the arrows, scar bar = 100μm (**B** and **C**) Semi-quantitative analysis performed by two independent pathologists showed that miR-106b significantly reduces RANKL expression and increases OPG expression. (**D**–**F**) Serum levels of RANKL and OPG and the RANKL:OPG ratio in each group. $^{*}P < 0.05$ compared with the sham group. $^{\#}P < 0.05$ compared with the NC group.

and decreased RANKL expression relative to the NC group. Meanwhile, miR-106b downregulation also suppressed the serum RANKL:OPG ratio and was associated with decreased osteoclast numbers and activity. However, the results presented herein differ from a previous study that found that miR-106b downregulation increases RANKL expression and promotes bone resorption in ovariectomized mice [18]. While there is no clear explanation for this variation, one report suggested that miR-106b inhibition has a positive effect on bone physiology and explained that variations in experimental conditions and models may contribute to such paradoxical results [28]. However, the distinct reason remains unidentified.

This study also showed that miR-106b inhibitor treatment significantly improves arthritis severity by reducing pro-inflammatory cytokine and inflammation-associated enzyme concentrations at the local and systemic levels when compared with the NC group. Previous studies have indicated that cytokines such as TNF-α, IL-1β and IL-6 are overexpressed in RA patient synovium and serum, thereby contributing to bone destruction in inflamed joints [1]. TNF-α and IL-1β stimulate fibroblast-like synoviocytes to secrete chemokines and MMPs, thus promoting inflammation-associated enzyme expression, such as COX-2 and inducible nitric oxide synthase, and inducing RANKL-mediated osteoclast activation [35]. Both TNF-α and IL-1β promote each other's expression, with TNF-α upregulating IL-1β production and IL-1β stimulating TNF-α expression. Interestingly, when IL-1β is missing, TNF-α stimulates inflammation responses but does not cause bone destruction in CIA mice [36]. IL-6 is not only a pro-inflammatory cytokine but also a stimulatory factor for osteoclastogenesis via indirect regulation of RANKL expression [37]. Therefore, TNF-α, IL-1β, IL-6 and COX-2 downregulation in the presence of miR-106b inhibitor may attenuate inflammatory responses and bone destruction in CIA mice.

Despite these interesting findings, there are certain limitations in our study. First, we did not investigate the mechanisms of action of miR-106b inhibition. Several genes involved in bone homeostasis have been identified as miR-106b targets [28]. In addition, miR-106b has also been shown to target the tumour suppressor gene phosphatase and tensin homologue [38], which is also critical for bone remodelling. Interestingly, RANKL is also a target for miR-106b [19]. Consistently we also observed a reduction of RANKL in miR-106b inhibitor treatment groups and subsequent decreased osteoclast formation and differentiation. This may partially explain the therapeutic effects of

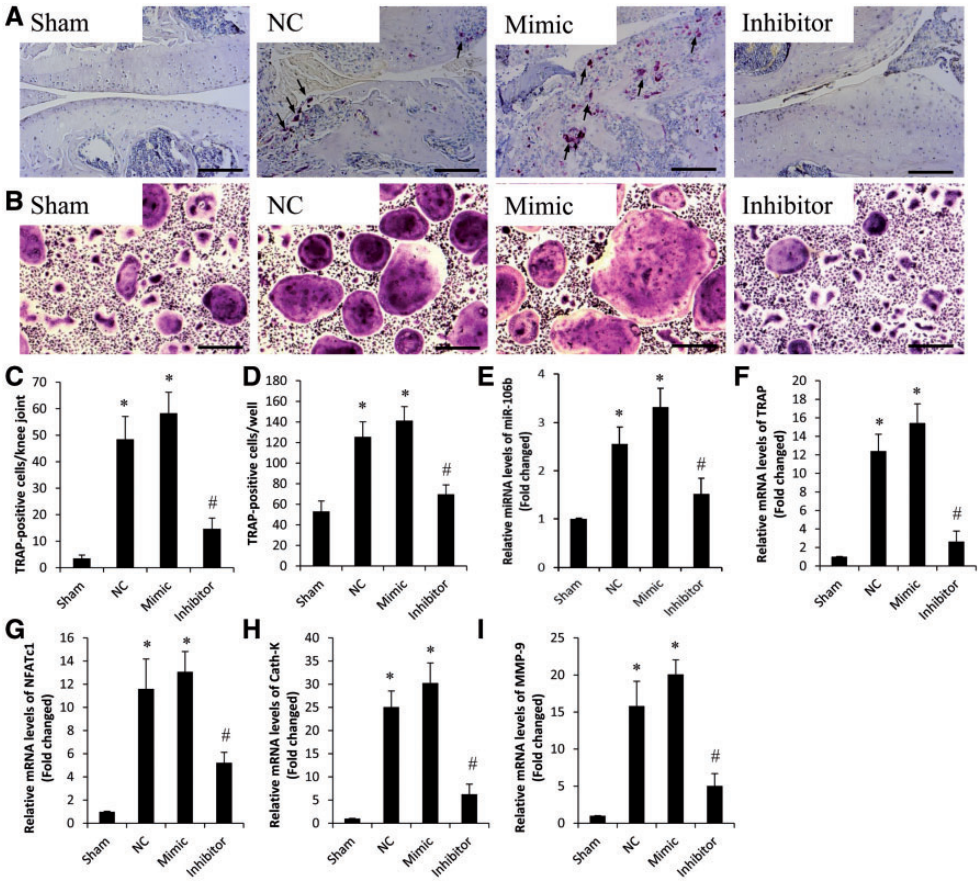
Fig. 5 miR-106b inhibition suppressed the expression of TNF-α, IL-1β, IL-6 and COX-2 in CIA mice



(**A**) Tissue sections of knee joints were immunohistochemically stained for TNF-α, IL-1β, IL-6 and COX-2 (brown, indicated by the arrows, scar bar = 100μm (**B**–**E**) Semi-quantitative analysis performed by two independent pathologists showed that miR-106b inhibition significantly reduced joint inflammation. (**F**–**H**) Serum levels of TNF-α, IL-1β and IL-6 in each group were determined by ELISA. $^{*}P < 0.05$ compared with the sham group. $^{\#}P < 0.05$ compared with the NC group.

miR-106b inhibition on RA-induced bone loss. Second, since bone destruction in RA is not only due to excessive bone resorption but also inhibition of bone formation, and because our results demonstrated that miR-106b inhibitor treatment significantly inhibited RA-induced bone loss through suppression of osteoclastic bone resorption and chronic inflammation, it remains unclear whether increased osteoblast differentiation and osteoblastic bone formation could have been involved in the protective effects of miR-106b inhibition on RA-induced bone loss. This question is currently the focus of ongoing studies in our laboratory.

FIG. 6 miR-106b inhibitor suppressed osteoclast formation



(**A**) Images of TRAP-stained knee sections for each group observed via light microscope (purple, indicated by the arrows, (scar bar = 100μm). (**B**) Bone marrow-derived monocyte/macrophage precursors (BMMs) obtained from CIA mice were incubated with M-CSF (30 ng/ml) and RANKL (50 ng/ml) for 5 days. TRAP staining was used to determine osteoclast formation and differentiation (scar bar = 500μm). (**C** and **D**) The number of TRAP-positive cells in knee sections or BMMs cultured were counted. (**E**–**I**) miR-106b, TRAP, NFATc1, Cath-K and MMP-9 gene expression examined via quantitative PCR. $^{*}P < 0.05$ compared with the sham group. $^{\#}P < 0.05$ compared with the NC group.

Conclusion

The results presented herein show that miR-106b is over-expressed in inflamed joints and is critically associated with the severity of arthritis in CIA mice. Moreover, miR-106b inhibition improved CIA-associated clinical and histological features, including attenuating bone destruction and restoring bone density by suppressing osteoclast formation and differentiation. Additionally, miR-106b inhibition exerts anti-inflammatory properties and an ability to regulate the RANKL:OPG ratio. These data suggest that miR-106b might be a promising therapeutic target for the treatment of human RA.

Funding: This research was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions and the National Nature Science Foundation of China (81101399, 81272018, 81301559, 81372018, 81472077 and 81572183).

Disclosure statement: The authors have declared no conflicts of interest.

References

1 Scott DL, Wolfe F, Huizinga TW. Rheumatoid arthritis. Lancet 2010;376:1094–108.

2 Boissier MC, Semerano L, Challal S, Saidenberg-Kermanac'h N, Falgarone G. Rheumatoid arthritis: from autoimmunity to synovitis and joint destruction. J Autoimmun 2012;39:222–8.

3 Fu J, Ling S, Liu Y *et al*. A small shared epitope-mimetic compound potently accelerates osteoclast-mediated bone damage in autoimmune arthritis. J Immunol 2013;191:2096–103.

4 Li J, Sarosi I, Yan XQ *et al*. RANK is the intrinsic hematopoietic cell surface receptor that controls

osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci USA 2000;97:1566–71.

5 Polzer K, Joosten L, Gasser J *et al*. Interleukin-1 is essential for systemic inflammatory bone loss. Ann Rheum Dis 2010;69:284–90.

6 Abou-Khalil R, Yang F, Mortreux M *et al*. Delayed bone regeneration is linked to chronic inflammation in murine muscular dystrophy. J Bone Miner Res 2014;29:304–15.

7 Straub RH, Cutolo M, Pacifici R. Evolutionary medicine and bone loss in chronic inflammatory diseases—a theory of inflammation-related osteopenia. Semin Arthritis Rheum 2015;45:220–8.

8 Zhu S, Pan W, Song X *et al*. The microRNA miR-23b suppresses IL-17-associated autoimmune inflammation by targeting TAB2, TAB3 and IKK-α. Nat Med 2012;18:1077–86.

9 Miyaki S, Sato T, Inoue A *et al*. MicroRNA-140 plays dual roles in both cartilage development and homeostasis. Genes Dev 2010;24:1173–85.

10 Nakamachi Y, Ohnuma K, Uto K *et al*. MicroRNA-124 inhibits the progression of adjuvant-induced arthritis in rats. Ann Rheum Dis 2016;75:601–8.

11 Zhou Q, Haupt S, Kreuzer JT *et al*. Decreased expression of miR-146a and miR-155 contributes to an abnormal Treg phenotype in patients with rheumatoid arthritis. Ann Rheum Dis 2015;74:1265–74.

12 Blüml S, Bonelli M, Niederreiter B *et al*. Essential role of microRNA-155 in the pathogenesis of autoimmune arthritis in mice. Arthritis Rheum 2011;63:1281–8.

13 Nakasa T, Shibuya H, Nagata Y, Niimoto T, Ochi M. The inhibitory effect of microRNA-146a expression on bone destruction in collagen-induced arthritis. Arthritis Rheum 2011;63:1582–90.

14 Zhang B, Xu CW, Shao Y *et al*. Evaluation and identification of microRNA-106 in the diagnosis of cancer: a meta-analysis. Int J Clin Exp Med 2014;7:3746–56.

15 Zhang J, Ren J, Chen H, Geng Q. Inflammation induced-endothelial cells release angiogenesis associated-microRNAs into circulation by microparticles. Chin Med J 2014;127:2212–7.

16 Tang H, Jiang H, Zheng J *et al*. MicroRNA-106b regulates pro-allergic properties of dendritic cells and Th2 polarisation by targeting early growth response-2 in vitro. Int Immunopharmacol 2015;28:866–74.

17 De Santis G, Ferracin M, Biondani A *et al*. Altered miRNA expression in T regulatory cells in course of multiple sclerosis. J Neuroimmunol 2010;226:165–71.

18 Guha D, Mancini A, Sparks J, Ayyavoo V. HIV-1 infection dysregulates cell cycle regulatory protein p21 in CD4+ T cells through miR-20a and miR-106b regulation. J Cell Biochem 2016;117:1902–12.

19 Wang T, Yin H, Wang J *et al*. MicroRNA-106b inhibits osteoclastogenesis and osteolysis by targeting RANKL in giant cell tumor of bone. Oncotarget 2015;6:18980–96.

20 Ni X, Xia T, Zhao Y *et al*. Downregulation of miR-106b induced breast cancer cell invasion and motility in association with overexpression of matrix metalloproteinase 2. Cancer Sci 2014;105:18–25.

21 Philippe L, Alsaleh G, Pichot A *et al*. MiR-20a regulates ASK1 expression and TLR4-dependent cytokine release in rheumatoid fibroblast-like synoviocytes. Ann Rheum Dis 2013;72:1071–9.

22 Lee SW, Kim JH, Park YB, Lee SK. Bortezomib attenuates murine collagen-induced arthritis. Ann Rheum Dis 2008;68:1761–7.

23 Brand DD, Latham KA, Rosloniec EF. Collagen-induced arthritis. Nat Protoc 2007;2:1269–75.

24 Garimella MG, Kour S, Piprode V *et al*. Adipose-derived mesenchymal stem cells prevent systemic bone loss in collagen-induced arthritis. J Immunol 2015;195:5136–48.

25 Bouxsein ML, Boyd SK, Christiansen BA *et al*. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. J Bone Miner Res 2010;25:1468–86.

26 Park MC, Kwon YJ, Chung SJ, Park YB, Lee SK. Liver X receptor agonist prevents the evolution of collagen-induced arthritis in mice. Rheumatology 2010;49:882–90.

27 Espinosa-Parrilla Y, Muñoz X, Bonet C *et al*. Genetic association of gastric cancer with miRNA clusters including the cancer-related genes MIR29, MIR25, MIR93 and MIR106: results from the EPIC-EURGAST study. Int J Cancer 2014;135:2065–76.

28 Fang T, Wu Q, Zhou L, Mu S, Fu Q. miR-106b-5p and mir-17-5p suppress osteogenic differentiation by targeting Smad5 and inhibit bone formation. Exp Cell Res 2016;347:74–82.

29 Luz-Crawford P, Ipseiz N, Espinosa-Carrasco G *et al*. PPARβ/δ directs the therapeutic potential of mesenchymal stem cells in arthritis. Ann Rheum Dis 2016;75:2166–74.

30 Le Goff B, Berthelot JM, Maugars Y, Heymann D. Osteoclasts in RA: diverse origins and functions. Joint Bone Spine 2013;80:586–91.

31 Sims NA, Green JR, Glatt M *et al*. Targeting osteoclasts with zoledronic acid prevents bone destruction in collagen-induced arthritis. Arthritis Rheum 2004;50:2338–46.

32 Geusens PP, Landewé RB, Garnero P *et al*. The ratio of circulating osteoprotegerin to RANKL in early rheumatoid arthritis predicts later joint destruction. Arthritis Rheum 2006;54:1772–7.

33 Knevel R, de Rooy DP, Saxne T *et al*. A genetic variant in osteoprotegerin is associated with progression of joint destruction in rheumatoid arthritis. Arthritis Res Ther 2014;16:R108.

34 Bossaller L, Rothe A. Monoclonal antibody treatments for rheumatoid arthritis. Expert Opin Biol Ther 2013;13:1257–72.

35 O'Gradaigh D, Ireland D, Bord S, Compston JE. Joint erosion in rheumatoid arthritis: interactions between tumour necrosis factor alpha, interleukin 1, and receptor activator of nuclear factor κB ligand (RANKL) regulate osteoclasts. Ann Rheum Dis 2004;63:354–9.

36 Zwerina J, Redlich K, Polzer K *et al*. TNF-induced structural joint damage is mediated by IL-1. Proc Natl Acad Sci USA 2007;104:11742–7.

37 Feng X, Shi Y, Xu L *et al*. Modulation of IL-6 induced RANKL expression in arthritic synovium by a transcription factor SOX5. Sci Rep 2016;6:32001.

38 Yang TS, Yang XH, Chen X *et al*. MicroRNA-106b in cancer-associated fibroblasts from gastric cancer promotes cell migration and invasion by targeting PTEN. FEBS Lett 2014;588:2162–9.