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# MicroRNA-124 inhibits TNF-α- and IL-6-induced osteoclastogenesis

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**Abstract**
Receptor activator for nuclear factor κB ligand (RANKL)-independent osteoclastogenic pathway was reported recently. MicroRNA (miR)-124 has been known to suppress RANKL-dependent osteoclastogenesis by inhibiting NFATc1 expression. However, whether miR-124 regulates a RANKL-independent pathway has not been elucidated. In this study, we examined whether a RANKL-independent pathway is regulated by miR-124 in addition to the RANKL-dependent one. Using osteoclastogenic culture and pit-formation assay, we found that a miR-124 mimic inhibited osteoclastogenesis in mouse bone marrow-derived macrophages stimulated by TNF-α, IL-6, and M-CSF in the presence of osteoprotegerin. We also showed that the expression levels of osteoclast-specific genes and NFATc1 protein were suppressed in the miR-124 mimic-transfected cells by performing quantitative-polymerase chain reaction and western blotting. Our results indicate that miR-124 is important in inhibiting both RANKL-dependent and -independent osteoclast differentiation by suppressing NFATc1-mediated pathway.

**Keywords** MicroRNA-124 · Rheumatoid arthritis · Osteoclast · Tumor necrosis factor-α · Interleukin-6 · RANKL-independent osteoclastogenesis

## Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic synovial inflammation and bone destruction [1, 2]. Osteoclasts are large, multinucleated cells formed by the differentiation and fusion of myeloid lineage precursor cells, and are thought to play a central role in arthritic bone destruction [3]. Receptor activator for nuclear factor κB ligand (RANKL), a member of the TNF cytokine superfamily, and M-CSF are key cytokines for osteoclastogenesis [4, 5]. RANKL specifically and strongly induces nuclear factor of activated T cell cytoplasmic 1 (NFATc1), which is the master regulator for osteoclast differentiation and is essential for the expression of osteoclast-specific genes [6]. Thus, the RANKL–NFATc1 axis has an important role in the bone destruction in RA.

In addition, roles of RANKL-independent pathways in osteoclastogenesis have also been reported [7]. Yokota's and William's group reported that the combination of TNF-α and IL-6 induces bone marrow macrophages to differentiate into osteoclast-like cells that have a bone-resorptive activity in vitro and in vivo [8, 9]. These studies showed that NFATc1 still played a key role in the RANKL-independent osteoclastogenesis pathway because inhibiting NFATc1 fully ameliorated the osteoclastogenesis. These results suggest that cytokines other than RANKL are involved in the inflammation and bone destruction observed in RA patients and that therapies targeting the downstream molecules of TNF-α and IL-6 signaling may be effective for preventing bone destruction in RA.

We previously demonstrated that the miRNA-124 level is significantly decreased in RA synoviocytes compared with osteoarthritis (OA) synoviocytes, and that miR-124 suppresses adjuvant-induced arthritis in rats, as demonstrated by decreases in synoviocyte proliferation, leucocyte

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infiltration, and cartilage and bone destruction [10]. Furthermore, miR-124 directly targets the 3′-UTR of human *NFATc1* mRNA, and suppresses the differentiation of human osteoclasts [11]. Lee et al. also demonstrated that miR-124 inhibits RANKL-induced osteoclastogenesis by suppressing NFATc1 and Rac1 in mice [12]. However, these studies examined the RANKL-dependent pathway, and the role of miR-124 in RANKL-independent osteoclastogenic differentiation has remained unclear. In this study, we aimed to evaluate the effects of miR-124 on the RANKL-independent osteoclastogenic pathway.

## Materials and methods

### Mice

C57BL/6 female mice (ages 6–10 weeks) were purchased from CLEA Japan. All animal experiments were carried out with the approval of the Animal Study Committee of Kobe University.

### Reagents and antibodies

Murine macrophage colony-stimulating factor (M-CSF), Osteoprotegerin (OPG), and IL-6 were purchased from R&D Systems. TNF-α was from Miltenyi Biotec. These cytokines were added for the stimulation of bone marrow macrophages (BMMs). Anti-NFATc1 antibody (clone; 7A6, Thermo Fisher Scientific) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody and anti-β-actin antibody (clone; C4, Santa Cruz) were used in Western blotting. MiR-124 mimic, which are small, chemically modified double-stranded RNAs that mimic endogenous miR-124, and negative control (negative mimic) were purchased from Thermo Fisher Scientific and added in the transfection experiment of BMMs.

### In vitro osteoclast differentiation assays and transfection of BMMs by miRNAs

Bone marrow cells (BMCs) were cultured in α-minimum essential medium (Gibco Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin (Gibco Invitrogen), and 20 ng/ml of M-CSF. Cells were cultured at $1 \times 10^5$ cells/well in 48-well plates and at $5 \times 10^6$ cells/dish in 6-cm dishes for the first 2 days (starting on day 0). After the initial 2-day culture, the BMCs were then used as bone marrow-derived macrophages (BMMs). Medium was changed to the fresh medium supplemented with 200 μg/ml of OPG, 50 ng/ml of TNF-α, and 50 ng/ml of IL-6, and the incubation was continued (starting on day 2). Half of the medium was replaced by fresh medium every other day until various assays were performed. The BMMs were transfected with miRNA mimics on day 4 using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific) and incubated for 4 h. The culture medium containing the transfection reagent was then replaced by fresh medium, and the cells continued to be incubated in the medium supplemented with OPG, TNF-α, and IL-6. For TRAP staining, the incubation was continued for another 4 days. Tartrate-resistant acid phosphatase (TRAP) was assayed with a TRAP Staining Kit (Wako Chemical) according to the manufacturer's instructions. We determined TRAP-positive and multinucleated cells (more than three nuclei) as osteoclasts.

### In vitro assay for resorption by osteoclasts

To measure the osteoclasts' resorptive function, a commercially available bone-resorption measurement kit (Bone Resorption Assay Kit 48; PG Research) was used. The osteoclasts were cultured as described above on a bone-resorption assay plate for 14 days in the presence of cytokines, which were replenished every other day. The cells were then removed and the wells were washed with 5% sodium hypochlorite. Images were captured with a BZ-X700 fluorescence microscope (Keyence) and analyzed using BZ-H3A software (Keyence).

### Real-time quantitative-polymerase chain reaction (qPCR) analysis

Small RNA-containing total RNA was isolated with a mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific), and complementary DNA (cDNA) was generated using the SuperScript® VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific). The qPCRs were performed using Fast SYBR Green Master Mix (Life Technologies) on a QuantStudio 7 Flex real-time PCR system (Thermo Fisher Scientific). The cDNA levels of each target gene were normalized to the housekeeping gene *HPRT*. Samples with no target amplification were assigned a value of zero. Primer sequences used in this study are listed in supplementary Table 1.

### Western blotting

Whole-cell lysates were extracted using CelLytic™ MT (Sigma-Aldrich). The concentrations of extracted protein were measured using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). The bound antibodies were visualized using a chemiluminescent reagent (GE Healthcare Life Sciences) following the manufacturer's instructions. The blots for β-actin served as a protein-loading control.

### Statistical analysis

One-way analysis of variance with Tukey's test for multiple comparisons of qPCR results and Kruskal–Wallis test with Dunn's post hoc test was performed using GraphPad PRISM software. Significance is indicated as follows: $*p < 0.05$ or $**p < 0.01$.

## Results

### Overexpression of a miR-124 mimic inhibits RANKL-independent osteoclastogenesis in vitro

To examine the regulatory role of miR-124 on the TNF-α/IL-6-induced osteoclastogenic pathway, mouse BMMs were transfected with a negative mimic or with a miR-124 mimic. Using qPCR, we confirmed that the miR-124 mimic was highly expressed in the miR-124 mimic-transfected BMMs 36 h after transfection (Supplementary Fig. 1). We also demonstrated that the overexpression of miR-124 mimic significantly decreased the number of TRAP-positive, multinucleated cells in the TNF-α/IL-6/OPG/M-CSF-induced osteoclasts (Fig. 1a, b). However, we found that there were few TRAP-positive cells harboring one or two nuclei, not determined as osteoclasts, in the miR-124 mimic-transfected wells. These results indicated that the miR-124 mimic suppressed the RANKL-independent TNF-α/IL-6-induced osteoclastogenesis in BMMs.

### Overexpression of a miR-124 mimic inhibits the bone-resorption capacity of osteoclasts

Next, we performed a bone-resorption assay to examine whether the miR-124 mimic transfection inhibited the bone-resorption capacity of differentiated osteoclasts. We found

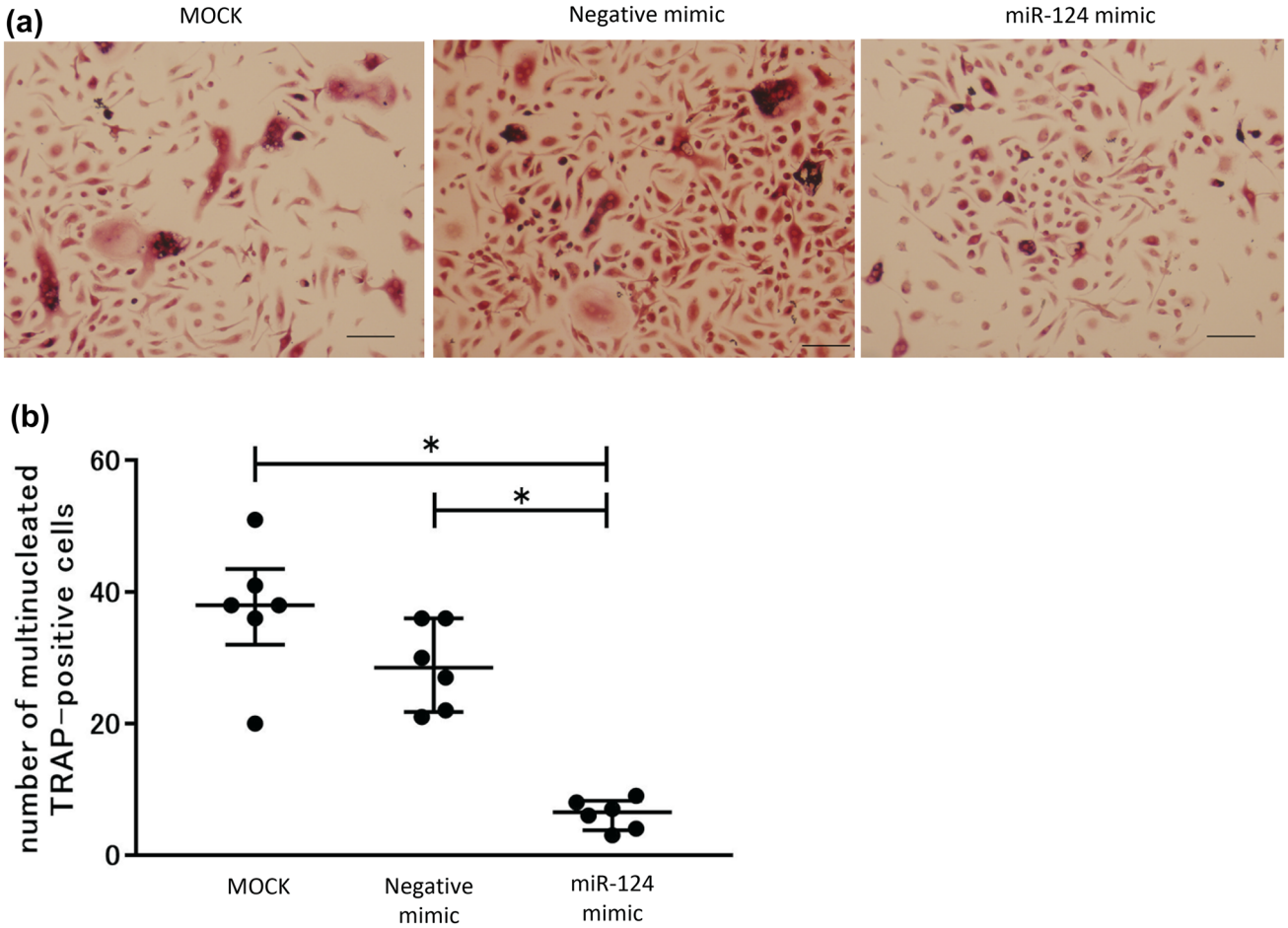


**Fig. 1** A MiR-124 mimic inhibits RANKL-independent osteoclastogenesis. **a** Mouse bone marrow cells were transfected with negative mimic or with miR-124 mimic, and cultured with M-CSF, IL-6, TNF-α, and OPG. The cells were fixed and stained for TRAP after 8 days of culture. Representative images of multinucleated TRAP-positive osteoclasts are shown (original magnification × 100). Scale bar indicates 100 μm. **b** Number of TRAP-positive multinucleated cells per well were counted. Data are shown as dot distributions. Interquartile ranges and medians were shown as horizontal lines. Representative data of three independent experiments are shown. $*p < 0.05$

that the miR-124 mimic transfection significantly inhibited the osteoclasts' pit-formation ability compared with mock- or negative mimic-transfected cells (Fig. 2). In addition, we found that the TRAP-staining-positive, mono-nucleated or di-nucleated cells generated in the presence of miR-124 mimic have little or no bone-resorption ability, and thus that bone resorption is inhibited by miR-124.

## MiR-124 mimic suppresses the expression of osteoclastogenic genes

To further elucidate the role of the miR-124 mimic in TNFα/IL-6-induced osteoclast differentiation, mouse BMMs were transfected with the negative mimic or the miR-124 mimic, and the mRNA expression levels of osteoclast-related markers were determined. The results from qPCR demonstrated that the transfection with the miR-124 mimic significantly decreased the expression levels of osteoclastogenic genes, such as *CtsK, ACP5, DC-stamp*, and *Itgb3* compared with the mock or negative mimic-transfected cells (Fig. 3). In contrast, the *NFATc1* mRNA expression level was similar in all groups (Fig. 3). These results suggested that miR-124 suppressed the TNF-α/IL-6-induced osteoclastogenesis by inhibiting the expression of osteoclast-specific genes.

## MiR-124 mimic inhibits the NFATc1 gene translation

We and others previously showed that miR-124 binds to the *NFATc1* mRNA 3′-UTR and inhibits the NFATc1 protein expression in $CD14^+$ monocytes or RAW264.7 cells [11, 12]. To examine the mechanism by which the miR-124 mimic inhibits TNF-α/IL-6-induced osteoclastogenesis, we studied the protein levels of NFATc1 in osteoclasts transfected with the miR-124 mimic. We found that the NFATc1 protein level was decreased in the miR-124 mimic-transfected cells compared to mock- and negative mimic-transfected cells (Fig. 4). Taken together, our results suggested

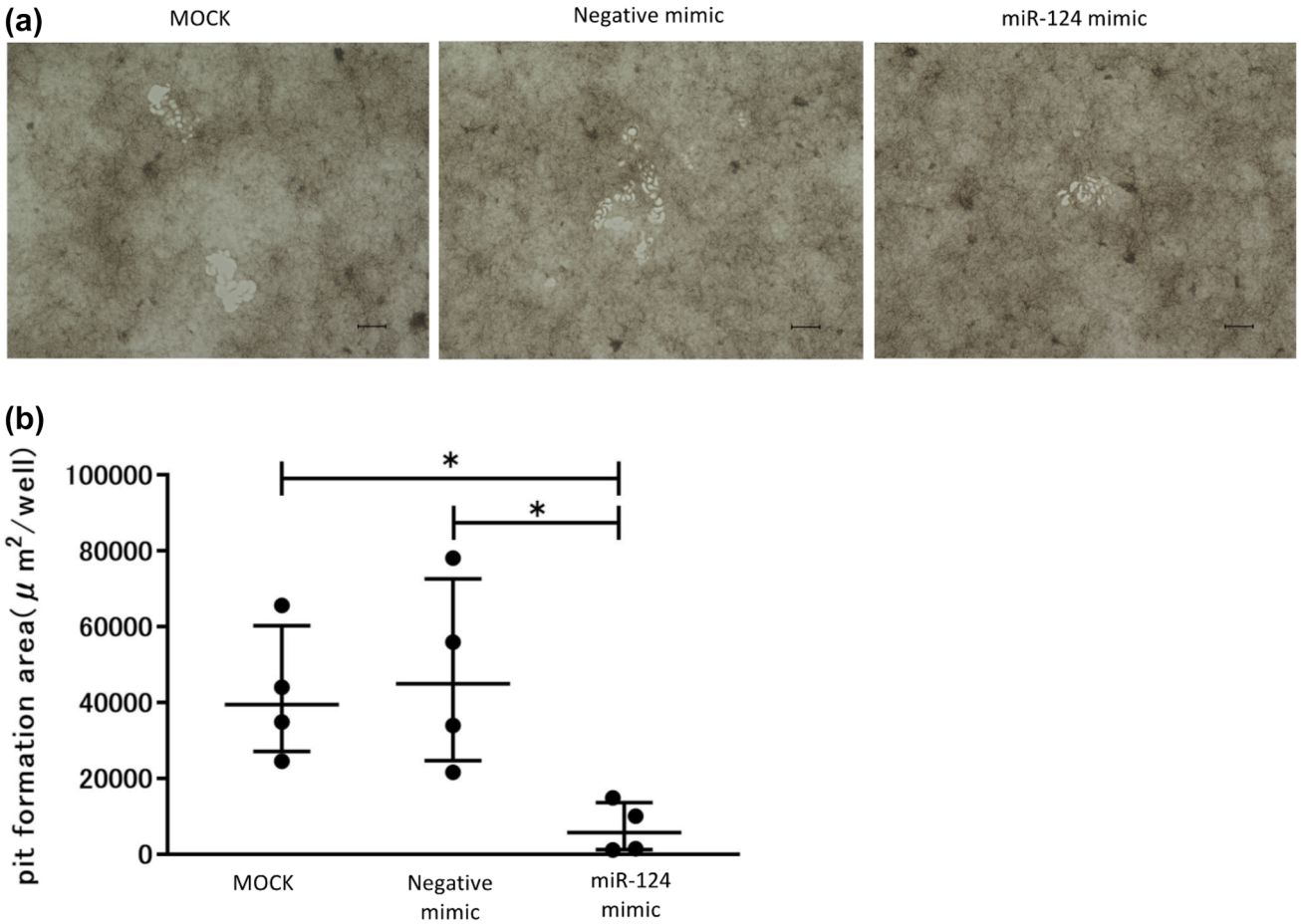


**Fig. 2** MiR-124 mimic inhibits the bone-resorption activity of osteoclasts. **a** Osteoclasts were generated on a bone-resorption assay plate with M-CSF, IL-6, TNF-α, and OPG for 14 days. Representative microscopic views of the resorption pits formed by the osteoclasts are shown (original magnification × 100). The scale bar indicates 100 μm. **b** The area of the resorption pits per well was quantified and expressed. Scale bar indicates 100 μm. Interquartile ranges and medians were shown as horizontal lines. $^*p < 0.05$

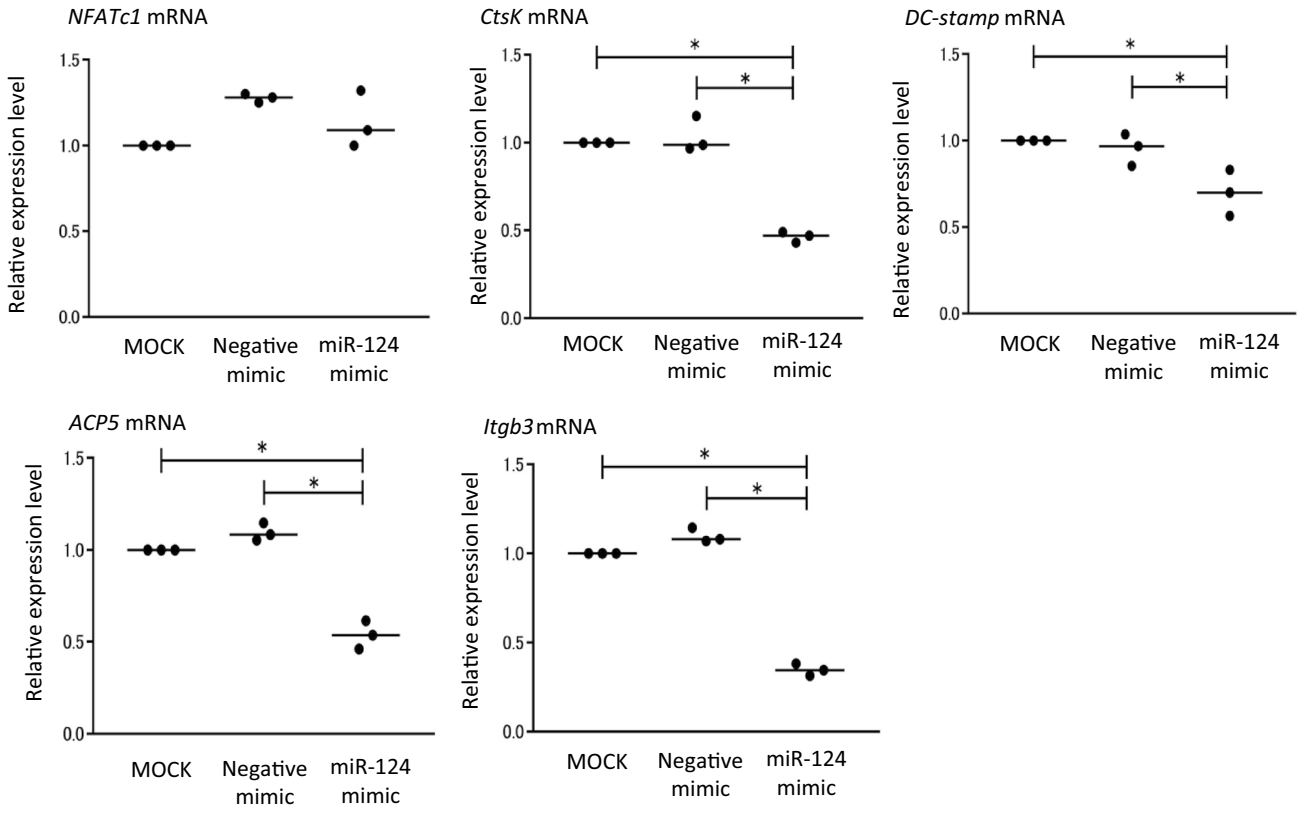


**Fig. 3** MiR-124 mimic inhibits osteoclastogenic gene expression. Mouse BMCs were transfected with negative mimic or miR-124 mimic, and cultured with medium supplemented with M-CSF, IL-6, TNF-α, and OPG for 36 h. The *NFATc1, CtsK, DC-stamp, ACP5*, and *Itgb3* mRNA levels were then determined using qPCR. *HPRT* was used as housekeeping gene. The relative expression level of each gene was determined by calculating ratio ΔΔCt of miR-124 mimic- or negative mimic-transfected cells to ΔΔCt of mock defined as 1.0. Data from three independent experiments are shown. $*p < 0.05$; $**p < 0.01$. *NFATc1* nuclear factor of activated T cells cytoplasmic 1, *CtsK* cathepsin K, *DC-stamp* dendritic cell-specific transmembrane protein, *ACP5* acid phosphatase-5, *Itgb3* integrin subunit beta 3

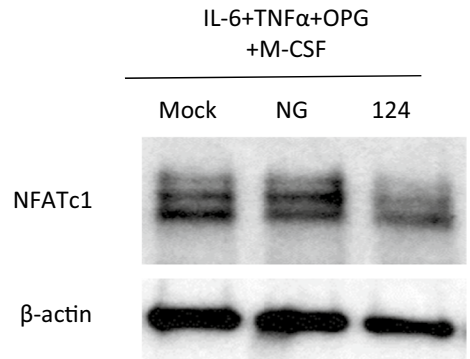


**Fig. 4** MiR-124 mimic decreases the expression of NFATc1 protein. Mouse BMCs were transfected with negative mimic or miR-124 mimic, and cultured with M-CSF, IL-6, TNF-α, and OPG for 2 days. Whole-cell lysates was prepared, and 10 μg of protein per well was subjected to SDS-PAGE. NFATc1 protein was determined and β-actin served as protein-loading control. *NFATc1* nuclear factor of activated T cell cytoplasmic 1

that the miR-124 mimic inhibits TNF-α/IL-6-induced NFATc1 translation, but not its transcription.

## Discussion

In this study, we found that a miR-124 mimic inhibited TNF-α/IL-6-dependent osteoclastogenesis and the expression of several osteoclast-specific genes by suppressing the translation of NFATc1 protein. We previously showed that miR-124 inhibits RANKL-induced osteoclastogenesis using human CD14-positive monocytes [11]. Lee et al. also demonstrated that miR-124 inhibits RANKL-induced osteoclastogenesis by suppressing NFATc1 and Rac1 in mice [12]. These results collectively indicate that miR-124 is a key regulator of osteoclastogenesis by targeting NFATc1 in both RANKL-dependent and RANKL-independent pathways.

RA is an autoimmune disease in which several factors act on the immune system, joints, and organs, causing chronic synovial or systemic inflammation and bone destruction. New treatments for RA that target specific

molecules such as cytokines, cytokine receptors, costimulatory molecules, and intracellular signaling molecules have provided therapeutic benefits and shed light on RA's pathogenic mechanisms [13–15]. In particular, TNF-α blockers are commonly used to treat RA. However, some patients do not respond to this treatment, due to the generation of anti-drug antibodies or to unknown mechanisms. The RANKL-independent TNF-α/IL-6-dependent osteoclast-like cells described by Yokota and William et al. emphasize the importance of regulating multiple cytokines in treating RA bone destruction and of investigating new strategies for inhibiting such RANKL-independent osteoclastogenesis pathways [8, 9].

A number of miRNAs are known to regulate bone-cell differentiation and function, bone development, and bone diseases [16]. The overexpression of pre-miR-223 precursors can completely inhibit the formation of TRAP-positive multinucleated cells by downregulating nuclear factor I A (NFI-A) [17]. In addition, Nakasa et al. found that expressing miR-146a inhibits osteoclastogenesis and that administering double-stranded miR-146a prevents joint destruction in arthritic mice [18]. In this paper, we showed that miR-124 inhibited osteoclastogenesis not only in the RANKL-dependent pathway but also in the RANKL-independent one by suppressing NFATc1, a critical molecule in osteoclastogenesis. Although the administration of these two miRNAs in arthritic mice also inhibits osteoclastogenesis, there is uniqueness in miR-124. First, miR-146a nor miR-223 is reported to control NFATc1 as their target molecule. Second, compared to miR-124, which is downregulated in RA synovium, both miR-146a and miR-223 are upregulated in RA synovium [19]. For miR-146a and miR-223, their enhanced expression in RA synovium may not be sufficient to suppress osteoclastogenesis effectively, and then the administration of extra high doses may be necessary to control osteoclastogenesis. For miR-124, on the contrary, its expression is suppressed in RA synovium, and then the release from its deficient status may be effective to inhibit osteoclastogenesis. Therefore, the concepts of efficacy are different between miR-124 and miR-146a/223 administration. Further investigations may be needed to understand the pathologic contribution of miRNAs which are upregulated or downregulated in RA. In accordance with our results that NFATc1 is important on RANKL-dependent and independent osteoclastogenesis, inhibitors of NFAT such as calcineurin inhibitors are therapeutic candidates for RA. In fact, cyclosporin and FK506 are used for RA drugs. Recently, Park et al. have reported that injection of an RCAN3 overexpression vector controlled arthritis development in mice with collagen-induced arthritis [20]. RCAN proteins have a highly conserved calcipressin inhibitor of calcineurin motif, and RCAN competes with NFATc1 for binding to calcineurin.

The biologics used to treat RA include antibodies against inflammatory cytokines and their receptors. These reagents are more expensive than conventional DMARDs, and some patients do not respond to them. Furthermore, inhibiting inflammatory cytokines might increase the risk for infectious diseases. Recently, miRNAs were studied as a potential treatment for viral hepatitis and pulmonary fibrosis [21, 22]. MiRNAs are considered more attractive than the existing molecule-targeting drugs such as antibodies because they have the potential to regulate multiple genes simultaneously. However, there are still some challenges to applying miRNAs therapeutically, such as delivery methods and safety. In this respect, Liu et al. developed a targeting system by conjugating eight repeating sequences of an aspartate ($D\text{-}Asp_8$) peptide with liposomes, which specifically delivered miRNA modulators to bone-resorption surfaces, without detectable liver or kidney toxicity in mice [23]. In addition, Brian et al. reported that intravenous delivery miRNAs in vivo inhibits osteoclast activity and reduces osteolytic bone metastasis [24]. Therefore, the development of delivery method is anticipated for the clinical application of miRNAs such as miR-124.

In summary, we discovered another role of miR-124 as a modulator of RANKL-independent osteoclastogenesis. Our results shed light on a possible target pathway for preventing bone destruction of RA.

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**Author contributions** Conceptualization: SK. Data curation and making figures and graphs: KO. Formal analysis: SK, JS. Investigation: YN, YN, KU. Methodology: SK, JS, SK. Integration of the study: KO, SK, JS, SK. Supervision: SK. Writing ± original draft: KO. Writing ± review and editing: SK, JS, SK.

## Compliance with ethical standards

**Conflict of interest** The authors declare no conflicts of interest. This study was approved by the Institutional Animal Care and Use Committee of Kobe University.

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