ORIGINAL ARTICLE

Expression of MicroRNAs in the Extracellular Microvesicles of Murine Osteoclasts

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SYNOPSIS
Bone is one of the most important oral tissues. Osteoclasts play important roles in bone tissue engineering. Previously, we profiled microRNA (miRNA) expression during osteoclastogenesis using microarrays. miRNAs are small, noncoding RNAs that are involved in various biological processes, including cellular differentiation, proliferation, apoptosis, and organ development. Recently, the presence of miRNAs in extracellular microvesicles was reported. It is not known whether osteoclasts secrete extracellular microvesicles containing miRNAs. We investigated miRNA expression in extracellular microvesicles in conditioned medium of cultured osteoclasts using RT-PCR. Specifically, we investigated eight miRNAs deemed important for osteoclastogenesis in our previous study: let-7e, miR-21, miR-33, miR-155, miR-210, miR-223, miR-378, and miR-1224. Of these, the expression levels of miR-378, miR-210, and miR-21 were very high, while no significant miR-33 or miR-1224 expression was detected. These results suggest that osteoclasts secrete extracellular microvesicles containing specific miRNAs, but that they do not contain the entire set of intracellular miRNAs.

***Key words**: MicroRNA, Extracellular Microvesicle, Exosome, Osteoclast*

INTRODUCTION

Bone is one of the most important oral tissues. It is maintained by the balance between bone formation and resorption[1, 2]. It is remodeled continuously by two specialized cell types: bone-forming osteoblasts and bone-resorbing osteoclasts [2]. Osteoclasts are tartrate-resistant acid phosphatase (TRAP)-positive, multinucleated giant cells that originate from the monocyte/macrophage lineage of hematopoietic stem cells[3]. Stromal cells and osteoblasts express macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL), which are essential for the induction of osteoclasts[1-3]. In terms of tissue engineering, osteoclasts and macrophages resorb scaffolds, making space for bone matrix formation[4]. Consequently, osteoclasts play an important role in bone regeneration.

We reported previously microRNAs (miRNAs) expression profiles during osteoclastogenesis using microarrays[5]. miRNAs are small, endogenous, noncoding RNAs of approximately 22 nu-

cleotides [1, 5]. miRNAs are incorporated into an RNA-induced silencing complex that contains argonaute-family proteins. The argonaute proteins recruit miRNAs specific for target mRNAs [1, 5], and the RNA-induced silencing complex either inhibits the translation of the target mRNAs or degrades the target mRNAs [1, 5]. Consequently, miRNAs are involved in the post-transcriptional regulation of mRNA function and participate in regulation of cellular differentiation, proliferation, apoptosis, and organ development [5].

Recently, miRNAs were reported to be present extracellular microvesicles and to function in other cells [6-9]. Extracellular microvesicles are small particles derived from the endosomal compartment[7, 9-11], and 40–100-nm-diameter microvesicles are defined as exosomes [11-13]. Extracellular microvesicles have an important role in cell-to-cell communication via the transfer of miRNAs, mRNAs, proteins, and bioactive lipids to target cells[6-13]. The secretion of microvesicles containing miRNAs depends on the cell type, biological conditions, and what kinds of miRNAs the cells contain [12, 13]. Although osteoclasts play important roles in bone remodeling and regeneration, it is not known whether osteoclasts secrete extracellular microvesicles containing miRNAs. Here, hypothesizing that osteoclasts secrete extracellular microvesicles containing miRNAs, we investigated the expression of miRNAs in extracellular microvesicles in conditioned medium of cultured osteoclasts using RT-PCR.

MATERIALS AND METHODS

1. Cell culture

All animal experiments were reviewed and approved by the Animal Use and Care Committee of Iwate Medical University (registration number 23-029). Five-week-old male ddY mice were purchased from SLC Japan (Hamamatsu, Japan). The mice were sacrificed, and their femurs and tibias were removed and dissected free of adherent soft tissue. The ends of the bones were cut, α-MEM (Invitrogen, Fredrick, MD, USA) was injected into one end of the bone, and the marrow cavity was slowly flushed to collect the marrow cells. Red blood cells were removed by treatment with phosphate-buffered saline (pH 7.2) containing 10 mM Tris and 0.83% NH_4Cl. The cells were seeded at a density of 2×10^5 cells/cm^2 and cultured in α-MEM containing 10% heat-inactivated fetal bovine serum (FBS) (Lot. 75300103; Moregate Biotech, Bulimba, Australia) and 10 ng/mL recombinant mouse macrophage colony-stimulating factor (M-CSF) (R&D Systems, Minneapolis, MN, USA) without antibiotics. After 2 days, the medium was changed, and the adherent cells were cultured in the presence of 10 ng/mL M-CSF and 100 ng/mL RANKL for up to an additional 82 h. The medium was exchanged after 48 h of RANKL/M-CSF treatment.

2. Extracellular microvesicle precipitation

Extracellular microvesicles exist not only in conditioned medium, but also in FBS. To measure the background expression of miRNAs, we isolated the microvesicles in FBS. After the culture period (RANKL/M-CSF treatment for 82 h), 6 mL each of conditioned medium treated with RANKL/M-CSF for 48 to 82 h and α-MEM containing 10% FBS without culturing for measurement of the background were collected. To pellet dead cells and cellular debris, the samples were centrifuged at 3,000 rpm for 5 min and 5 mL of each supernatant was transferred to a fresh tube. Next, 1 mL of ExoQuick-TC™ Exosome Isolation Reagent (System Biosciences, Mountain View, CA, USA) was added to each tube, and the samples were incubated over-

night at 4°C. The next day, they were centrifuged at 3,000 rpm for 30 min to pellet the microvesicles. The supernatant was removed, and the pellets containing microvesicles were isolated.

3. Quantitative RT-PCR (qRT-PCR) analysis of mRNA and miRNA expression

Total RNA was harvested from bone marrow macrophages that had been treated with RANKL/M-CSF for 0, 24, or 82 h, and the RNA was isolated from the pellets of microvesicles as described above using a mirVana™ miRNA isolation kit (Ambion, Austin, TX, USA). To measure mRNA expression, the RNA was reverse-transcribed using PrimeScript RT reagent kit (Takara Bio, Otsu, Japan). The resulting cDNAs were then amplified using SYBR Premix EX *Taq* II (Takara Bio) with gene-specific primers (Takara Bio) (Table 1). The target gene expression in each sample was normalized to the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) signal. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression levels. To evaluate miRNA expression, the RNA was reverse-transcribed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with miRNA-specific primers (Applied Biosystems), as described previously[5]. The expression of mature miRNAs was analyzed using appropriate TaqMan miRNA assays (Applied Biosystems). The intracellular expression was quantified using snoRNA 202 as an endogenous control[14,15]. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative miRNA expression levels.

4. TRAP staining

After culture, the cells were fixed with formalin/acetone/citrate (10% / 65% / 25%) for 1 min at room temperature and stained for TRAP using an acid phosphatase assay kit (Sigma-Aldrich, St. Louis, MO, USA), as described previously[5].

5. Statistical analysis

Data are presented as means ± standard deviation (SD). For multiple-group comparisons, one-way analysis of variance (ANOVA) was performed. For two independent groups, unpaired two-tailed Student's *t*-tests were used, assuming unequal variance, to identify statistically significant differences (* $P < 0.05$; ** $P < 0.01$).

RESULTS

1. Bone marrow macrophages differentiated into osteoclasts

We cultured murine bone marrow cells treated with M-CSF for 2 d. Of the M-CSF treated cells, the adherent cells are defined as bone marrow macrophages (BMMs). The BMMs differentiated into TRAP-positive multinucleated giant cells; *i.e.*, osteoclasts, after treat-

Table 1 Primer pairs used in qRT-PCR analyses.

Primer	GenBank Acc.	Forward (5' - 3') Reverse (5' - 3')
CtsK	NM_007802.3	CAGCAGAACGGAGGCATTGA CTTTGCCGTGGCGTTATACATACA
Gapdh	NM_008084.2	TGTGTCCGTCGTGGATCTGA TTGCTGTTGAAGTCGCAGGAG
Nfatc1	NM_198429.1	AGGTGGCAATGAGCAGTCTGTG TCCAGAGGTAGACCCTGATGGAAG

ment with RANKL and M-CSF (RANKL/M-CSF) for 82 h (Fig. 1 a). Nuclear factor of activated T cells, cytoplasmic 1 (*Nfatc1*), which is a master regulator of osteoclastogenesis, was upregulated after RANKL/M-CSF treatment for 82 h (Fig. 1 b). The osteoclast-specific gene *Cathepsin K* (*CtsK*) strongly expressed after treatment with RANKL/M-CSF for 82 h (Fig. 1 c). Therefore, we used this culture system to analyze the intra- and extracellular miRNA expression in osteoclasts.

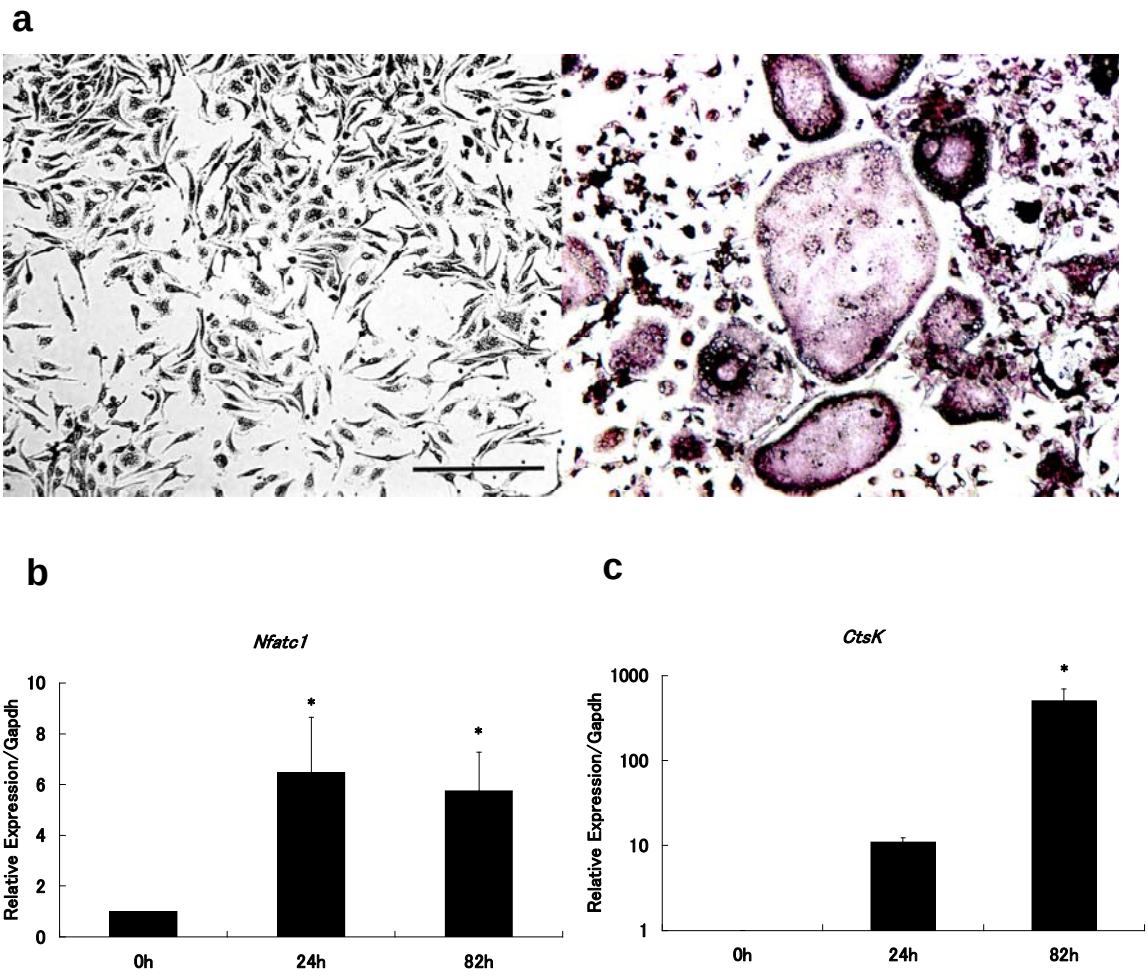

Figure 1 Differentiation of osteoclasts from murine bone marrow macrophages.
Murine bone marrow macrophages (BMMs) were incubated for 82 h with RANKL (100 ng/mL) and M-CSF (10 ng/mL); osteoclasts formed after this treatment.
a; Representative microscopic images of BMMs before (left panel) and after (right panel) 82 h stimulation with RANKL and M-CSF and stained for TRAP. Bar = 50 μm.
b and c; Relative expression of the osteoclast genes *Nuclear factor of activated T cells, cytoplasmic 1* (*Nfatc1*) (b) and *Cathepsin K (CtsK*) (c) in BMMs treated with RANKL and M-CSF for 0, 24, or 82 h, as measured by qRT-PCR. The values are relative to 1 at 0 h (before stimulation) for each mRNA. The target gene expression in each sample was normalized to the *Gapdh* signal. The experiments were performed in three times. The data are presented as means ± SD. One-way ANOVA was performed (* $P < 0.05$ *vs*. 0 h).

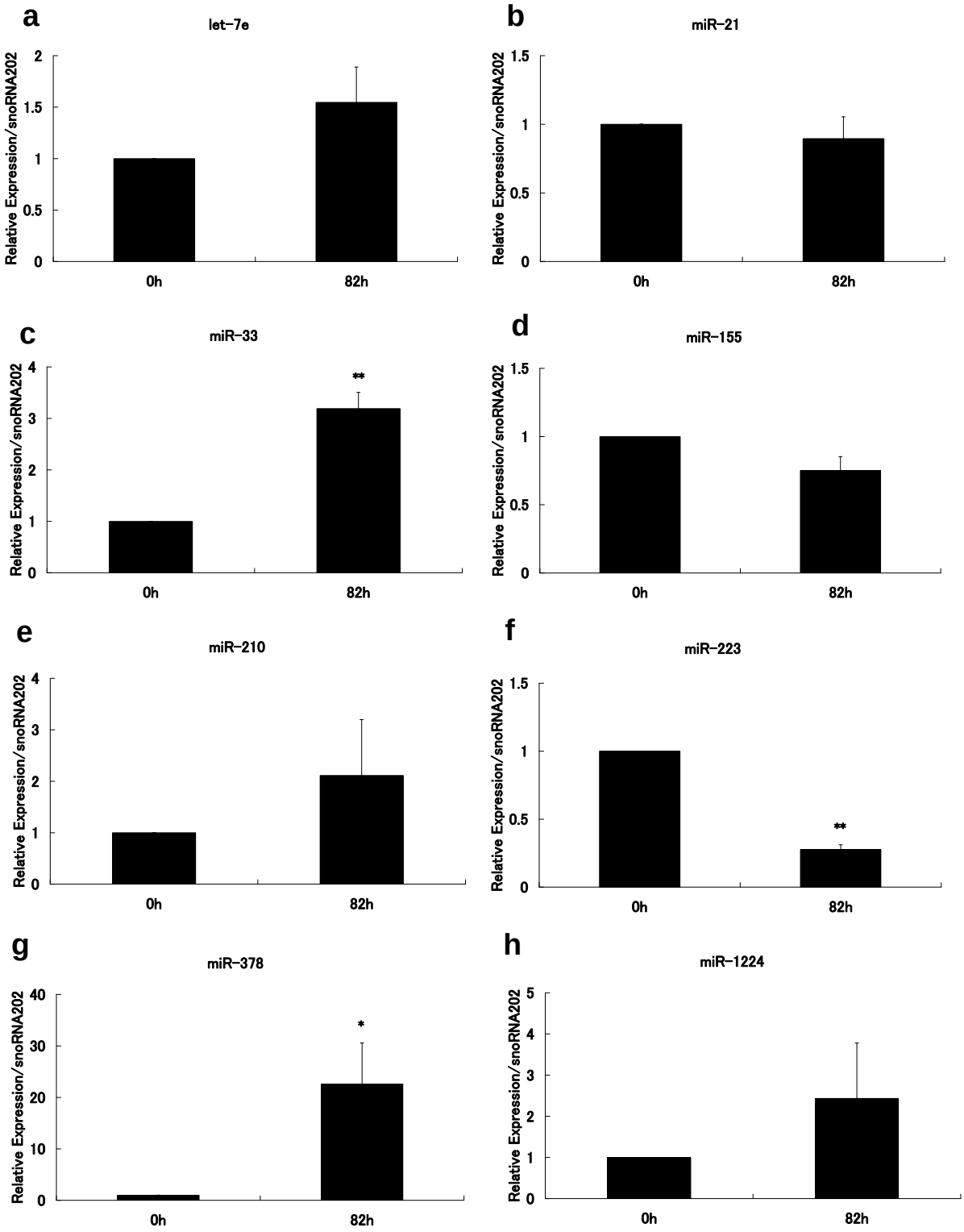

Figure 2 Intracellular microRNA expression in BMMs during osteoclastogenesis.
Murine bone marrow macrophages (BMMs) were cultured in the presence of RANKL (100 ng/mL) and M-CSF (10ng/mL) for 0 or 82 h, and then subjected to qRT-PCR. The expression levels of let-7e (a), miR-21 (b), miR-33 (c), miR-155 (d), miR-210 (e), miR-223 (f), miR-378 (g), and miR-1224 (h) are shown. The values are relative to 1 at 0 h (before stimulation) for each microRNA. Quantification was performed using snoRNA 202 as an endogenous control. The experiments were performed in three times. The data are presented as means ± SD. Unpaired two-tailed Student's *t*-tests were used, assuming unequal variance, to identify statistically significant differences (* $P < 0.05$ *vs*. 0 h, ** $P < 0.01$ *vs*. 0 h).

2. The expression of intracellular miRNAs in osteoclasts

We reported global intracellular miRNA expression profiling during osteoclast differentiation[5]. Based on that study, we selected eight miRNAs that we consider important for osteoclastogenesis: let-7e, miR-33, miR-155, miR-210, miR-223, miR378, and miR-1224. The expression levels of let-7e, miR-33, miR-210, miR378, and miR-1224 were upregulated compared with before RANKL/ M-CSF treatment (Fig. 2 a, c, e, g, and h), while those of miR-155 and miR-223 were downregulated compared with before RANKL/M-CSF treatment (Fig. 2 d and f). Despite the downregulation of miR-155 and miR-223, the expression baselines of these miRNAs were relatively high (data not shown). The level of miR-21 expression did not change significantly in RANKL/M-CSF-treated BMMs (Fig. 2 b); the baseline expression was relatively high (data not shown).

3. The expression of miRNAs in the extracellular microvesicles of osteoclasts

We next evaluated the expression of miRNAs in the extracellular microvesicles of osteoclasts. Of the eight miRNAs we investigated, miR-378 was expressed to the greatest degree in the extracellular microvesicles (Fig. 3 g), followed by miR-210 and miR-21 (Fig. 3 b and e). miR-155 and miR-223 were highly expressed in the extracellular microvesicles (Fig. 3 d and f), and let-7e expression was detected (Fig. 3 a). No significant miR-33 or miR-1224 expression was detected in the extracellular microvesicles (Fig. 3 c and h). These results are similar to those obtained from qRT-PCR amplification curves after elimination of the background miRNA expression in FBS (data not shown).

DISCUSSION

For the first time, we demonstrated that osteoclasts secrete extracellular microvesicles containing specific miRNAs. Of the eight miRNAs we investigated, miR-378 was expressed at the highest level in the extracellular microvesicles. We previously postulated that miR-378 plays an important role in osteoclastogenesis and its intracellular expression increased during osteoclast differentiation, with a 21-fold increase in RANKL/ M-CSF-treated BMMs[5]. It is reported that miR-378 promotes cell survival and participates in blood vessel formation[16]. Since bone tissue is highly vascular, miR-378 in the microvesicles might promote cell survival and blood vessel formation around osteoclasts.

In microvesicles, miR-210 showed the second-highest expression; its intracellular expression in BMMs also increased after RANKL/M-CSF treatment. miR-210 regulates various genes involved in the cell cycle, differentiation, development, membrane trafficking, and migration / adhesion[17, 18]. In microvesicles, miR-210 might play various roles in cell-to-cell communication. The level of miR-21 expression in the microvesicles was higher than that of let-7e, miR-155, or miR-223. miR-21 is one of the most abundant miRNAs detected in microvesicles circulating in the blood of cancer patients[19]. Considering both our results and these reports, both cancer cells and normal cells, such as osteoclasts, might secrete miR-21-containing microvesicles into the circulation.

We detected high-level miR-223 expression in the microvesicles. Although the intracellular expression of miR-223 decreased in RANKL / M-CSF-treated murine BMMs, the expression baseline was relatively high. miR-223 is highly expressed in myeloid and bone marrow cells[20]. miR-223 plays a key role in osteoclastogenesis[1, 5, 21], and the expression of intracellular miR-223

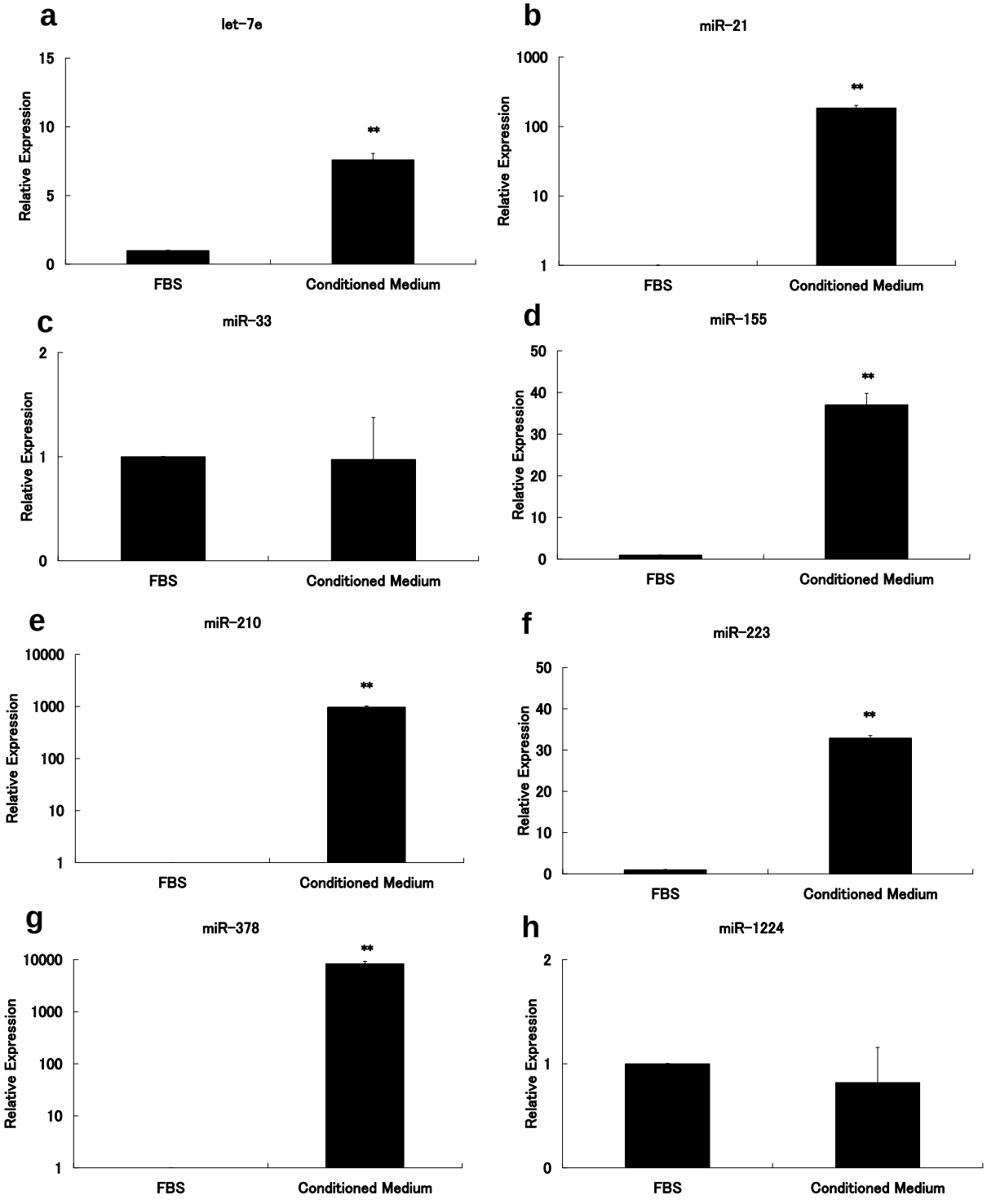

Figure 3 MicroRNA expression in the extracellular microvesicles in osteoclasts.
Murine bone marrow macrophages (BMMs) were cultured in the presence of RANKL (100 ng/mL) and M-CSF (10 ng/mL) for 82 h. RNA was isolated from extracellular microvesicles in the RANKL/M-CSF-treated conditioned medium after 48 to 82 h, and then subjected to qRT-PCR. The expression levels of let-7e (a), miR-21 (b), miR-33 (c), miR-155 (d), miR-210 (e), miR-223 (f), miR-378 (g), and miR-1224 (h) are shown. The values are relative to 1 for α-MEM containing 10% FBS without culturing for each microRNA. The experiments were performed in three times. The data are presented as means ± SD. Unpaired two-tailed Student's *t*-tests were used, assuming unequal variance, to identify statistically significant differences (** $P < 0.01$ *vs*. FBS).

decreases during osteoclast differentiation[5]. The overexpression of miR-223 blocks osteoclast differentiation, whereas the inhibition of miR-223 expression has the reverse effect[21]. Since the expression of miR-223 increases incrementally as granulocytes mature[20], it might affect granulocyte maturation around osteoclasts in the bone marrow. miR-155 expression was downregulated in RANKL/M-CSF-treated murine BMMs during osteoclast differentiation. Active macrophage differentiation involves the rapid upregulation of miR-155 expression, which leads to the suppression of RANKL-induced osteoclastogenesis[22]. If the miR-155 in the extracellular microvesicles functions similarly, it might inhibit osteoclast differentiation via negative feedback. Let-7e expression was also detected in the microvesicles. Identified let-7e targets include cell cycle regulators such as CDC25A and CDK6[23]. Thus let-7e within microvesicles might be involved in regulation of the cell cycle of other cells.

No significant miR-33 or miR-1224 expression was detected in the extracellular microvesicles. However, the intracellular expression of these miRNAs was upregulated during osteoclast differentiation. Our results suggest that the entire set of intracellular miRNAs is not present in the extracellular microvesicles of osteoclasts.

Here, we demonstrated that osteoclasts secrete extracellular microvesicles containing let-7e, miR-21, miR-155, miR-210, miR-223, and miR-378. It is widely known that growth factors play important roles in tissue engineering[24]. In future, extracellular microvesicles might be useful as new growth factors in oral tissue engineering.

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