miR-92a-3p encapsulated in bone metastatic mammary tumor cell–derived extracellular vesicles modulates mature osteoclast longevity

Norihisa Uehara¹ | Yukari Kyumoto-Nakamura¹ | Yoshikazu Mikami² | Manabu Hayatsu² | Soichiro Sonoda¹ | Takayoshi Yamaza¹ | Akiko Kukita³ | Toshio Kukita¹

¹Division of Oral Biological Sciences, Department of Molecular Cell Biology & Oral Anatomy, Faculty of Dental Science, Kyushu University, Fukuoka, Japan
²Division of Microscopic Anatomy, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
³Department of Microbiology, Faculty of Medicine, Saga University, Saga, Japan

Correspondence
Norihisa Uehara, Division of Oral Biological Sciences, Department of Molecular Cell Biology & Oral Anatomy, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Email: ueharan@dent.kyushu-u.ac.jp

Funding information
Japan Science and Technology Agency, Grant/Award Number: JPMJCR19H3; JSPS KAKENHI, Grant/Award Number: 15K11014 and 18K09522

Abstract
Aberrant osteoclast formation and activation are the hallmarks of osteolytic metastasis. Extracellular vesicles (EVs), released from bone metastatic tumor cells, play a pivotal role in the progression of osteolytic lesions. However, the mechanisms through which tumor cell–derived EVs regulate osteoclast differentiation and function have not been fully elucidated. In this study, we found that 4T1 bone metastatic mouse mammary tumor cell–derived EVs (4T1-EVs) are taken up by mouse bone marrow macrophages to facilitate osteoclastogenesis. Furthermore, treatment of mature osteoclasts with 4T1-EVs promoted bone resorption, which was accompanied by enhanced survival of mature osteoclasts through the negative regulation of caspase-3. By comparing the miRNA content in 4T1-EVs with that in 67NR nonmetastatic mouse mammary tumor cell–derived EVs (67NR-EVs), miR-92a-3p was identified as one of the most enriched miRNAs in 4T1-EVs, and its transfer into mature osteoclasts significantly reduced apoptosis. Bioinformatic and Western blot analyses revealed that miR-92a-3p directly targeted phosphatase and tensin homolog (PTEN) in mature osteoclasts, resulting in increased levels of phospho-Akt. Our findings provide novel insights into the EV-mediated regulation of osteoclast survival through the transfer of miR-92a-3p, which enhances mature osteoclast survival via the Akt survival signaling pathway, thus promoting bone resorption.

KEYWORDS
apoptosis, breast cancer, extracellular vesicles, miRNA, osteoclast

Abbreviations: 3′-UTRs, 3′-untranslated regions; BMMs, bone marrow macrophages; CTSK, cathepsin K; EVs, extracellular vesicles; miRNAs, microRNAs; MMP-9, matrix metalloproteinase-9; NFATc1, nuclear factor of activated T cells c1; NTA, nanoparticle-tracking analysis; PTEN, phosphatase and tensin homolog; RANKL, receptor activator of nuclear factor-kappa B ligand; siRNA, small interfering RNA; TRAP, tartrate-resistant acid phosphatase.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2022 The Authors. Cancer Science published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.
Bone homeostasis is maintained by the orchestrated actions of bone-forming osteoblasts and bone-resorbing osteoclasts. The continuous action of these specialized cells allows for appropriate bone remodeling and calcium homeostasis throughout life. However, once tumor cells colonize and grow in the bone microenvironment, physiological bone metabolism is disrupted. Osteolytic metastases are frequently found in patients with advanced stages of cancers, including breast, lung, and kidney cancers, which are often accompanied by severe bone pain, fracture, and hypercalcemia due to aberrant osteoclastogenesis and bone resorption.

In these lesions, tumor cells develop a symbiotic relationship with bone-resident cells to establish a more favorable environment to grow, known as a “vicious cycle.” In this cycle, cancer cells secrete local factors, such as parathyroid hormone-related protein, interleukins, and prostaglandin E2, which increase the expression of RANKL in bone marrow stromal cells and osteoblasts to stimulate osteoclast formation and bone resorption. These events result in the release of bone matrix-derived growth factors, such as transforming growth factor-β1 (TGF-β1) and insulin-like growth factor I (IGF-I), further promoting tumor cell growth. Thus, the appearance of aberrantly activated osteoclasts, induced via intercellular communication between tumor cells and bone microenvironments, leads to osteolytic bone destruction.

Extracellular vesicles (EVs) are lipid bilayer membrane vesicles that are shed by most cells and key mediators of intercellular communication. EVs include exosomes and microvesicles. Exosomes, generated via the endosomal pathway, are typically 30–150 nm in diameter. Microvesicles, 100–1000 nm in diameter, are shed by plasma membrane budding. Although EVs vary in size and intracellular origin, they affect cell behavior when taken up by neighboring or distant cells through their bioactive molecules, such as microRNAs (miRNAs), mRNAs, DNAs, lipids, and proteins.

Recent studies have revealed that EVs derived from osteoclasts, osteoblasts, and their precursors are involved in bone remodeling, especially in osteoclast and osteoblast differentiation. Furthermore, EVs from bone metastatic breast cancer cells modulate osteoclastogenesis and function by transferring miRNAs and proteins. MiRNAs are small noncoding RNA molecules that bind to the 3′-untranslated regions (3′-UTRs) of target mRNAs to induce translational repression or mRNA degradation. MiRNAs play critical roles in various developmental processes and physiological and pathological conditions. Although the contribution of EVs to cancer metastasis is evident, the role of cancer cell-derived EVs and their role in the regulation of osteoclast differentiation and function during osteolytic metastasis needs to be elucidated.

In this study, we investigated the effects of bone metastatic mammary tumor cell–derived EVs on the regulation of osteoclast differentiation and function.
instructions (Thermo Fisher Scientific). Bone marrow macrophages (BMMs) were seeded at $5 \times 10^4$ per well on 24-well plates in α-MEM containing 10% FBS in the presence of M-CSF (20 ng/ml). The next day, the cells were incubated with the PKH67-labeled EVs for 24 h. The cells were stained with Hoechst33342 (Dojindo), and images were captured using a BZ-8100 fluorescence microscope (Keyence).

### 2.5 In vitro osteoclastogenesis

Osteoclasts were generated as previously described. Briefly, BMMs prepared from the femurs and tibiae of 5-week-old male ddY mice (Japan SLC) were seeded at $1.5 \times 10^4$ cells per well on 96-well plates and cultured in αMEM supplemented with 10% FBS, 20 ng/ml M-CSF (Wako) and 50 ng/ml RANKL (Oriental Yeast) in the presence of 100 ng/ml EVs for 4 days. The cells were fixed and stained using the Acid Phosphatase Leukocyte Kit (Sigma). The numbers of tartrate-resistant acid phosphatase (TRAP)-positive multinuclear osteoclasts (>three nuclei) per well were counted.

### 2.6 Bone resorption assay

In vitro osteoclastogenesis was performed in a mouse BMM culture system using RepCell temperature-responsive cell culture-ware (CellSeed Inc.). BMMs were seeded at $4 \times 10^5$ cells per 35-mm RepCell dish in αMEM supplemented with 10% FBS, 20 ng/ml M-CSF, and 50 ng/ml RANKL. On day 4 of osteoclast differentiation, osteoclasts were detached from a 35-mm RepCell dish by placing the dishes at 4°C for 15 min. Detached osteoclasts were collected by centrifugation and seeded on three dentin discs (Wako) in 96-well plates in osteoclastogenic medium in the presence of 100 ng/ml EVs and cultured for 7 days. Half of the culture medium in the presence of 100 ng/ml EVs was replaced every 3 days. The cells were then removed using a 10% bleach solution and cleaned using ultrasonication. Bone resorption pits were stained with horseradish peroxidase–conjugated wheat germ agglutinin (WGA)-lectin (Sigma-Aldrich) and detected using 3, 3′-diaminobenzidine tetrahydrochloride (Wako). Images were captured, and the resorption pit areas were determined using the BZ analyzer software (Keyence).

### 2.7 Apoptosis assay

Bone marrow macrophages were seeded at $1.5 \times 10^5$ cells per well on 96-well plates and cultured in osteoclastogenic medium for 4 days to differentiate. Osteoclasts were then cultured in osteoclastogenic medium containing 100 ng/ml EVs for up to 2 days. The osteoclast apoptosis rate was expressed as the number of morphologically intact TRAP-positive multinucleated cells. Osteoclast apoptosis was confirmed using the Cytotoxicity LDH Assay Kit (Dojindo).

### 2.8 Statistical analysis

All discrete values are expressed as the mean±standard deviation (SD) from at least three independent experiments. Data were analyzed using a paired t test for comparison between two groups or one-way analysis of variance (ANOVA) followed by Tukey’s test for multiple comparisons. Statistical significance was set at p < 0.05.

Additional detailed descriptions of materials and methods are in Supplemental Methods.

### 3 RESULTS

#### 3.1 Characterization of mouse mammary tumor cell–derived EVs

Results of the NTA (Figure 1A) indicated that several peaks ranging in size from 40 to 500 nm were identified in EVs from both 67NR (67NR-EVs) and 4T1 (4T1-EVs) cells with mean particle sizes...
of 134.3 and 125.0 nm, respectively. Electron microscopy revealed that both 67NR-EVs and 4T1-EVs showed a typical rounded exosomal morphology (Figure 1A, inset). Western blot analysis showed that exosome markers, such as CD9, CD63, and Alix, were abundant in both 67NR-EVs and 4T1-EVs compared with those in cell lysates (Figure 1B). In addition, albumin was also detected in both 67NR-EVs and 4T1-EVs as non-EV structures. We further examined whether 67NR-EVs and 4T1-EVs can be taken up by mouse BMMs by treating BMMs with PKH67-labeled EVs derived from 67NR and 4T1 cells. The results showed a punctate pattern of green fluorescence in the cytoplasm of BMMs treated with either 67NR-EVs or 4T1-EVs (Figure 1C).

3.2 | Bone metastatic tumor cell–derived EVs facilitate osteoclastogenesis

Treatment of BMMs or RAW264.7 cells with 4T1-EVs resulted in a significant increase in the number of TRAP-positive MNCs compared with those in 67NR-EVs or untreated controls (Figure 2A), which coincided with an increase in the expression levels of osteoclast differentiation marker genes, including TRAP, cathepsin K (Ctsk), mmp-9, and calcitonin receptor (Cacr; Figure 2B). Furthermore, we examined the expression level of nfatc1 and c-fos, fundamental transcription factors involved in osteoclastogenesis. Interestingly, 4T1-EV treatment led to a marked increase in the levels of nfatc1 and c-fos on day 4, whereas 67NR-EV treatment did not affect the expression level, which was the same as in the untreated control. To ascertain the osteoclastogenic effect of 4T1-EVs, exosome release in 4T1 cells was inhibited by Rab27a small interfering RNA (siRNA; Figure 2D), resulting in a decrease in the exosome amount in the culture supernatant (Figure 2E). Osteoclastogenesis was examined by TRAP staining after coculture of BMMs with Rab27a knockdown 4T1 cells (Figure 2F). Exosome release inhibition in 4T1 cells significantly decreased the number of TRAP-positive osteoclasts (Figure 2G), confirming the involvement of 4T1-EVs in osteoclastogenesis.

3.3 | Bone metastatic tumor cell–derived EVs promote bone resorption

4T1-EV treatment led to a marked increase in the resorbed area, whereas 67NR-EV treatment did not lead to changes in the bone resorption area compared with that of the untreated control (Figure 3A). Active osteoclasts are characterized by the expression of proteinases, which are essential for bone resorption. To determine if the 4T1-EV-induced increase in the resorbed area may be due to changes in protease expression, we analyzed the mRNA and protein levels of cathepsin K and MMP-9 (Figure 3B,C). No differences were observed in the mRNA expression levels of cathepsin K and MMP-9 in mature osteoclasts treated with 4T1-EVs and 67NR-EVs (Figure 3B). Similarly, 4T1-EV treatment did not affect the protein levels of the active forms of cathepsin K (~23kDa) and MMP-9 (lower band: ~86kDa; Figure 3C), suggesting that the 4T1-EV–induced bone resorption promotion was independent of the alteration of the expression or activation of cathepsin K and MMP-9.

3.4 | Bone metastatic tumor cell–derived EVs inhibit mature osteoclast apoptosis

Mature osteoclasts treated with 4T1-EVs maintained morphologically intact osteoclasts compared with those treated with 67NR-EVs or the untreated control (Figure 4A). Similarly, the LDH assay confirmed a decrease in cell death in mature osteoclasts treated with 4T1-EVs (Figure 4B). To determine the mechanism underlying the 4T1-EV–induced maintenance of osteoclast survival, the protein levels of pro- and antiapoptotic molecules were examined using Western blot analysis. No significant changes were observed in the protein levels of Bcl-2 and Bcl-XL between the treatment groups (Figure 4C, left panel), whereas 4T1-EV–treated mature osteoclasts exhibited a marked decrease in the protein levels of cleaved caspase-3 and Bim compared with those treated with 67NR-EVs or the untreated control (Figure 4C, right panel). These results indicate that 4T1-EVs enhanced mature osteoclast survival by downregulating caspase-3 and Bim, resulting in bone resorption promotion.

3.5 | Identification of miRNAs specifically expressed in bone metastatic tumor cell–derived EVs

To identify miRNAs specifically expressed in 4T1-EVs, we compared miRNAs in 4T1-EVs with those in 67NR-EVs. We identified 15 upregulated (Table 1) and 25 downregulated miRNAs in 4T1-EVs (Table S1). Interestingly, four of the 15 miRNAs, miR-92a-3p, miR-17-5p, miR-20a-5p, and miR-18-5p, were components of MIR17HG (miR-17-92 cluster genes), reported to be oncogenes in many types of cancer, including metastatic breast cancer cells. As it is unclear if the miRNAs encapsulated in 4T1-EVs contribute to osteoclast differentiation and function, we next focused our investigation on miR-92a-3p, the most abundant miRNA in 4T1-EVs. Both the intracellular and extracellular expression levels of miR-92a-3p were verified in 67NR and 4T1 cells using qPCR, and miR-92a-3p was specifically expressed in 4T1-EVs and 4T1 cells (Figure 5A). We also examined the intracellular and extracellular expression levels of miR-92a-3p in different molecular subtypes and the metastatic potential of human breast cancer cell lines defined as luminal A (MCF-7 and T47D) and triple-negative (MDA-MB-231, MDA-MB-436, and BT-549) breast cancer. Similar to the intracellular and extracellular expression levels of miR-92a-3p in 67NR and 4T1 cells, the expression of miR-92a-3p was significantly higher in both cells and EVs of MDA-MB-231 and MDA-MB-436, with increased in vivo metastatic potential compared with that of nonmetastatic MCF-7 and T47D cells (Figure 5B).
FIGURE 2  Metastatic mammary tumor cell–derived extracellular vesicles (EVs) facilitate osteoclastogenesis. (A) Bone marrow macrophages and RAW264.7 cells were differentiated into osteoclasts. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), and TRAP-positive multinuclear cells (≥3 nuclei) were counted. (B, C) mRNA expression of osteoclast differentiation–related molecules were analyzed using quantitative real-time PCR. For each target gene, the expression level was normalized to that of ß-2-microglobulin and shown relative to that of the EV (−) control (1) for comparison. (D) Rab27a protein level in 4T1 cells transfected with Rab27a siRNA or control siRNA examined by Western blot. (E) Amounts of EVs in the culture supernatant of Rab27a-knockdown (KD) and control 4T1 cells determined by ELISA. (F) Design of the coculture system for osteoclastogenesis. (G) RAW264.7 cells were differentiated into osteoclasts cocultured with Rab27a-KD or control 4T1 cells. TRAP-positive multinuclear cells (≥3 nuclei) were counted. Data represent the mean±SD from three independent experiments. *p < 0.05; **p < 0.01 using ANOVA with Tukey’s test for (A), (B), and (C), and Student’s t test for (E) and (F).
To elucidate the role of miR-92a-3p in osteoclastogenesis and osteoclast survival, miR-92a-3p mimics, inhibitors, or NC miRNAs were transfected into BMMs or mature osteoclasts. The increased level of miR-92a-3p was confirmed after the transfection of miR-92a-3p mimics in BMMs and mature osteoclasts (Figures 5C and 6C). Although miR-92a-3p tended to increase osteoclastogenesis (Figure 5D), the increase was not as significant as that observed following 4T1-EV treatment (Figure 2A). We next examined the effect of miR-92a-3p expression on mature osteoclast longevity. The results showed that miR-92a-3p significantly promoted osteoclast survival, as evidenced by the maintenance of intact osteoclasts (Figure 5E) and the release of LDH in the culture medium (Figure 5F). These results suggest that miR-92a-3p, enriched in 4T1-EVs, is likely associated with osteoclast survival signaling.

3.7 | MiR-92a-3p regulates cell survival signaling by directly targeting PTEN

To investigate the mechanism by which miR-92a-3p regulates mature osteoclast survival, potential miR-92a-3p targets were analyzed using bioinformatic tools, including miRDB and Targetscan. PTEN was among the genes predicted to be...
targeted by miR-92a-3p. Specifically, a binding site was found in the 3’UTR of PTEN mRNA. PTEN is an important protein phosphatase that regulates various cellular processes, including osteoclast differentiation and cell survival through the PI3K-Akt pathway.\(^{22,23}\) To verify the binding of miR-92a-3p to PTEN 3’UTR, we performed a luciferase reporter assay using luciferase reporter constructs containing wild-type (wt) or mutant (mut) PTEN 3’UTR (Figure 6A). The miR-92a-3p mimic significantly decreased the luciferase reporter activity of the wt PTEN 3’UTR-containing vector compared with that of vectors containing the control miRNA. This suppressive effect was abolished when the luciferase reporter vector containing mut PTEN 3’UTR was used (Figure 6A), indicating that PTEN is a direct target of miR-92a-3p. Western blot analysis further revealed that the PTEN protein levels were significantly decreased in mature osteoclasts transfected with miR-92a-3p mimics compared with those of cells transfected with miR-92a-3p inhibitors or control miRNA (Figure 6B), which inversely correlated with enhanced Akt phosphorylation (Figure 6C,D). Similar to miR-92a-3p transfection into mature osteoclasts, decreased PTEN protein levels and increased Akt phosphorylation were detected after treating mature osteoclasts with 4T1-EVs (Figure 6E–G), indicating that 4T1-EVs regulate osteoclast survival signaling through the miR-92a-3p/PTEN/Akt axis.

4 | DISCUSSION

Osteolytic bone metastases are a common complication in advanced breast cancer.\(^{24}\) The appearance of aberrantly activated osteoclasts induced via intercellular communication between metastasized tumor cells and bone microenvironments leads to osteolytic bone destruction.\(^{3}\) Previous studies indicated that tumor cell–derived EVs are key mediators of intercellular communication between metastasized tumor cells and bone microenvironments\(^{25,26}\), however, how they regulate osteoclast differentiation and function has not yet been fully elucidated. In this study, we demonstrated that 4T1-EVs promote osteoclast differentiation. Furthermore, we found that mature osteoclast treatment with 4T1-EVs rendered osteoclasts less prone to apoptosis, resulting in an increase in bone resorption. Moreover, we are the first to provide evidence that miR-92a-3p is enriched in 4T1-EVs and that it plays an important role in regulating mature osteoclast survival by targeting PTEN (Figure 7).

Despite accumulating evidence linking mammary tumor cell–derived EVs to bone metastatic process,\(^{25}\) the precise mechanisms through which cancer cell–derived EVs regulate bone microenvironments are still under investigation. In our in vitro studies, BMM and RAW264.7 cell treatment with 4T1-EVs markedly facilitated RANKL-induced osteoclastogenesis, compared with 67NR-EV treatment. This finding was likely due to the ability of 4T1-EVs to enhance RANKL signaling through the upregulation of two fundamental transcription factors, nfatc1 and c-fos (Figure 2C). RANKL is a major physiological and pathological regulator of osteoclast differentiation and functions. Loftus et al.\(^{11}\) reported that MDA-MB-231 osteolytic human breast cancer cell–derived EVs can induce osteoclastogenesis in the absence of RANKL. In contrast, 4T1-EVs could not induce osteoclastogenesis in RAW264.7 cells without RANKL stimulation (data not shown). Furthermore, we confirmed that exosome secretion inhibition by Rab27a knockdown in 4T1 cells decreased RANKL-induced osteoclastogenesis in RAW264.7 cells (Figure 2D–G). These results suggest that the stimulatory effect of 4T1-EVs on osteoclastogenesis is, at least partly, due to the cooperative action of RANKL signaling.

Although studies demonstrated that bone metastatic tumor cell–derived EVs affect osteoclast differentiation,\(^{25}\) few studies addressed their roles in bone resorption by mature osteoclasts. Mature osteoclast treatment with 4T1-EVs on dentin slices resulted in a significant increase in bone resorption. Bone-resorbing osteoclasts are characterized by acid secretion and the expression of proteinases, such as V-ATPase, TRAP, cathepsin K, and MMP-9.\(^{18}\) Despite the marked increase in bone resorption in 4T1-EV–treated mature osteoclasts, no significant change in the mRNA levels of Ctsk and mmp-9 was detected, nor in the protein levels of the active form of cathepsin K and MMP-9 (Figure 3). The ability of osteoclasts to resorb bone also depends on mature osteoclast longevity. Gallet et al. reported that conditioned media from MDA-MB-231 human breast cancer cells inhibited osteoclast apoptosis, resulting in increased bone resorption.\(^{27,28}\) Similarly, we found that 4T1-EV treatment reduced mature osteoclast apoptosis, accompanied by decreased caspase-3 and Bax protein levels (Figure 4), indicating that 4T1-EVs may render mature osteoclasts less prone to apoptosis, resulting in enhanced bone resorption.

Recent studies on breast cancer have revealed that several miRNAs contained in EVs contribute to osteoclast differentiation and osteolytic metastasis.\(^{24}\) Wu et al.\(^{29}\) showed that bone-tropic

---

**TABLE 1** Upregulated microRNAs (miRNAs) in 4T1 extracellular vesicles (4T1-EVs) compared with those in 67NR-EVs

| miRNA          | mirBase accession no. | Fold change |
|----------------|------------------------|-------------|
| mmu-miR-92a-3p | MIMAT0000539           | 25.28       |
| mmu-miR-6931-5p| MIMAT0027762           | 14.52       |
| mmu-miR-132-3p | MIMAT000144            | 8.17        |
| mmu-miR-17-5p  | MIMAT000649            | 5.78        |
| mmu-miR-130a-3p| MIMAT000141            | 4.50        |
| mmu-miR-5126   | MIMAT002637            | 4.32        |
| mmu-miR-125b-5p| MIMAT000136            | 3.92        |
| mmu-miR-93-5p  | MIMAT000540            | 3.39        |
| mmu-miR-1224-5p| MIMAT0005460           | 3.16        |
| mmu-miR-151-3p | MIMAT000161            | 2.79        |
| mmu-miR-8110   | MIMAT0031416           | 2.75        |
| mmu-miR-25-5p  | MIMAT0017049           | 2.39        |
| mmu-miR-20a-5p | MIMAT000529            | 2.38        |
| mmu-miR-7040-5p| MIMAT0027984           | 2.20        |
| mmu-miR-18a-5p | MIMAT000528            | 2.10        |
**FIGURE 5** Effects of miR-92a-3p on osteoclast formation and survival. (A) Quantitative real-time PCR analysis of the miR-92a-3p levels in 67NR and 4T1 cells and extracellular vesicles (EVs) isolated from the respective culture supernatant. (B) Quantitative real-time PCR analysis of the miR-92a-3p levels in human breast cancer cells and EVs isolated from the respective culture supernatant. (C) miR-92a-3p expression levels in bone marrow macrophages (BMMs) upon transfection with miR-92a-3p mimics, inhibitors, and control microRNAs (miRNAs). (D) Representative images of tartrate-resistant acid phosphatase (TRAP) staining and quantification of TRAP-positive multinuclear cells. BMMs were transfected with the miR-92a-3p mimic, inhibitor, and miRNA control and then cultured in the presence of M-CSF and RANKL for 4 days. (E) Representative images of TRAP staining and quantification of defective osteoclasts. Mature osteoclasts were transfected with the miR-92a-3p mimic, inhibitor, and miRNA control and then cultured in the presence of M-CSF and RANKL for 2 days. (F) Mature osteoclast apoptosis was evaluated using the LDH assay. Data represent the mean ± SD from three independent experiments. *p < 0.05; **p < 0.01 using ANOVA with Tukey’s test.
estrogen receptor–positive breast cancer cell–derived exosomal miR-19a and integrin-binding sialoprotein enhanced the differentiation of osteoclasts and bone metastasis. Another study also demonstrated that exosomal miR-20a-5p released by MDA-MB-231 cells was transferred to BMMs and facilitated osteoclastogenesis via downregulation of SRC kinase signaling inhibitor 1 (SRCN). In our miRNA array analysis, we identified 15 miRNAs that were specifically enriched in 4T1-EVs compared with those in 67NR-EVs. Intriguingly, four of these miRNAs (miR-92a-3p, miR-17-5p, miR-20a-5p, and miR-18-5p) belong to the miR-17-92 cluster, which...
is overexpressed in various human cancers and is upregulated in serum exosomes from patients with advanced breast cancer.\textsuperscript{19,30} We found that miR-92a-3p was the most abundant miRNA in both cells and EVs of 4T1-EVs. miR-92a-3p expression is significantly increased in the plasma exosomes and breast tissues from patients with triple-negative breast cancer.\textsuperscript{30,31} Similarly, our results confirmed that miR-92a-3p was upregulated in triple-negative breast cancer cells, such as MDA-MB-231 and MDA-MB-436, which indicated the metastatic potential of miR-92a-3p in breast cancer cells.

In vitro osteoclastogenesis assays demonstrated that transfection of miR-92a-3p in BMMs tended to increase the number of TRAP-positive osteoclasts, but was less effective in osteoclast formation, as was observed following 4T1-EVs treatment (Figure 2A). The expression level of miR-92a-3p in BMMs treated with 4T1-EVs (Figure S1) was lower than that in BMMs after transfection of miR-92a-3p mimic. Several reports showed that proteins, such as L-plastin, peroxiredoxin 4,\textsuperscript{12} and amphiregulin,\textsuperscript{32} which are present in several types of tumor cell-derived EVs, stimulate osteoclast differentiation. Other reports showed that cancer cell-derived exosomal miRNAs, such as miR-17-5p and miR-20a-5p, also promote osteoclast differentiation.\textsuperscript{30} These were identified as upregulated miRNAs in 4T1-EVs in our study (Table 1). Therefore, our results suggest that the introduction of miR-92a-3p alone was less effective than 4T1-EV treatment in RANKL-induced osteoclastogenesis, and that other miRNAs, mRNAs, and/or proteins contained in 4T1-EVs may also participate in the regulation of osteoclastogenesis. Further investigation is required to clarify how 4T1-EVs stimulate osteoclastogenesis and survival.

Next, we examined the role of miR-92a-3p in mature osteoclast longevity. After transfecting mature osteoclasts with miR-92a-3p mimics, we observed a significant decrease in apoptotic osteoclasts. To delineate the molecular mechanisms involved in miR-92a-3p-mediated survival signals in mature osteoclasts, we performed bioinformatic analysis. As a potential target, the putative binding site of miR-92a-3p was present in the 3' UTR of murine PTEN. Its direct binding to PTEN 3' UTR was confirmed using a luciferase reporter gene assay. PTEN was reported to be involved in osteoclast differentiation and cell survival signaling through Akt phosphorylation.\textsuperscript{22,23} PTEN knockdown increases Akt phosphorylation, resulting in the promotion of osteoclast formation.\textsuperscript{22} Conversely, PTEN overexpression in RAW264.7 cells blocked RANKL-induced osteoclastogenesis and induced apoptosis.\textsuperscript{23} Our results demonstrate that transfection with the miR-92a-3p mimic decreased the PTEN protein levels. Furthermore, we confirmed that treatment of mature osteoclasts with 4T1-EVs decreased the PTEN levels, which were negatively correlated with the protein levels of caspase-3 and Bax, suggesting that miR-92a-3p regulates mature osteoclast survival through the transfer of 4T1-EVs.

This study has some limitations. Our conclusion is based on the evidence obtained by in vitro osteoclastogenesis and bone resorption models, and only one cell line was used. Further investigations are needed to elucidate the mechanisms by which tumor cells develop a symbiotic relationship with bone-resident cells, including osteoblasts, to establish more favorable environments via EVs. In conclusion, our findings provide a novel regulatory mechanism of the mature osteoclast lifespan by which the osteolytic mammary tumor cell EV-derived miR-92a-3p post-transcriptionally inhibits PTEN, activating cell survival signaling. Thus, miR-92a-3p may be a potential therapeutic target and prognostic marker for patients with osteolytic bone metastasis.

**ACKNOWLEDGMENTS**

This study was supported by JSPS KAKENHI Grant Numbers 18K09522 and 15K11014 (NU) and partially supported by JST, CREST Grant Number JPMJCR19H3 (YM).

**DISCLOSURE**

The authors declare no conflict of interest.

**ETHICS STATEMENT**

Approval of the research protocol by an Institutional Reviewer Board N/A.

**INFORMED CONSENT**

N/A.

**REGISTRY AND THE REGISTRATION NO. OF THE STUDY/TRIAL**

N/A.

**FIGURE 7** Hypothetical model of the role of 4T1 extracellular vesicles (4T1-EVs) and miR-92a-3p in osteoclast differentiation and function modulation. Uptake of 4T1-EVs by bone marrow macrophages results in the marked promotion of osteoclastogenesis through nfATC1 and c-fos upregulation. To promote bone resorption, miR-92a-3p in 4T1-EVs inhibits mature osteoclast apoptosis by targeting PTEN resulting in Akt phosphorylation.
ANIMAL STUDIES

Animal experiments were performed in accordance with guidelines and procedures approved by the Institutional Animal Care and Use Committee of Kyushu University (protocol number: A19-270-0).

REFERENCES

1. Clarke B. Normal bone anatomy and physiology. Clin J Am Soc Nephrol. 2008;3(Suppl 3):S131-S139.
2. Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer. 2002;2:584-593.
3. Weilbaecher KN, Guise TA, McCauley LK. Cancer to bone: a fatal attraction. Nat Rev Cancer. 2011;11:411-425.
4. Roodman GD. Mechanisms of bone metastasis. J Bone Oncol. 2016;5:93-95.
5. van Niel G, D’Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol. 2018;19:213-228.
6. Abels ER, Breakefield XO. Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake. Cell Mol Neurobiol. 2016;36:301-312.
7. Gao M, Gao W, Papadimitriou JM, Zhan C, Gao J, Zheng M. Exosomes-the enigmatic regulators of bone homeostasis. Bone Res. 2018;6:36.
8. Xie Y, Chen Y, Zhang L, Ge W, Tang P. The roles of bone-derived exosomes and exosomal microRNAs in regulating bone remodeling. J Cell Mol Med. 2017;21:1033-1041.
9. Guo L, Zhu Y, Li L, et al. Breast cancer cell-derived exosomal miR-20a-5p promotes the proliferation and differentiation of osteoclasts by targeting SRCIN1. Cancer Med. 2019;8:5687-5701.
10. Loftus A, Cappariello A, George C, et al. Extracellular vesicles from osteotropically breast cancer cells affect bone resident cells. J Bone Miner Res. 2020;35:396-412.
11. Tiedemann K, Sadvakassova G, Mikolajewicz N, et al. Exosomal release of L-plastin by breast cancer cells facilitates metastatic bone osteolysis. Transl Oncol. 2019;12:462-474.
12. Yuan X, Qian N, Ling S, et al. Breast cancer exosomes contribute to pre-metastatic niche formation and promote bone metastasis of tumor cells. Theranostics. 2021;11:1429-1445.
13. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281-297.
14. Uehara N, Kyumoto- Nakamura Y, Yamaza T, Yasuda H, Fukita K. Osteoblast-derived Laminin-332 is a novel negative regulator of osteoclastogenesis in bone microenvironments. Lab Invest. 2017;97:1235-1244.
15. Delaisse JM, Andersen TL, Esgis MT, Henriksen K, Troen T, Blavier L. Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclastic activities. Microsc Res Tech. 2003;61:504-513.
16. Mogilyansky E, Rigoutsos I. The miR-17-92/2 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. Cell Death Differ. 2013;20:1603-1614.
17. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife. 2015;4:e05005.
18. Chen Y, Wang X. mirDB: an online database for prediction of functional microRNA targets. Nucleic Acids Res. 2020;48:D127-D131.
19. Jang HD, Noh JY, Shin HJ, Lin JI, Lee SY. PTEN regulation by the Akt/GSK-3beta axis during RANKL signaling. Bone. 2013:55-126-131.
20. Suganagi T, Alvarez U, Hruska KA. PTEN regulates RANKL- and osteopontin-stimulated signal transduction during osteoclast differentiation and cell motility. J Biol Chem. 2003;278:5001-5008.
21. Kozlow W, Guise TA. Breast cancer metastasis to bone: mechanisms of osteolysis and implications for therapy. J Mammary Gland Biol Neoplasia. 2005;10:169-180.
22. Giannandrea D, Citro L, Lesma E, Bignotto M, Platonova N, Chiararamote R. Restoring tissue homeostasis at metastatic sites: a focus on extracellular vesicles in bone metastasis. Front Oncol. 2021;11:644109.
23. Li S, Wang W. Extracellular vesicles in tumors: a potential mediator of bone metastasis. Front Cell Dev Biol. 2021;9:639514.
24. Gallet M, Montaverti R, Sevnet N, Brazier M, Kamel S. Ability of breast cancer cell lines to stimulate bone resorbing activity of mature osteoclasts correlates with an anti-apoptotic effect mediated by macrophage colony stimulating factor. Apoptosis. 2006;11:1909-1921.
25. Gallet M, Sevnet N, Dupont C, Braier M, Kamel S. Breast cancer cell line MDA-MB 231 exerts a potent and direct anti-apoptotic effect on mature osteoclasts. Biochem Biophys Res Commun. 2004;319:690-696.
26. Wu K, Feng J, Luy F, et al. Exosomal miR-19a and IBSP cooperate to induce osteolytic bone metastasis of estrogen receptor-positive breast cancer. Nat Commun. 2021;12:5196.
27. Moi L, Braaten T, Al-Shibli K, Lund E, Busund LR. Differential expression of the miR-17-92 cluster and miR-17 family in breast cancer according to tumor type; results from the Norwegian women and cancer (NOWAC) study. J Transl Med. 2019;17:334.
28. Shin VY, Siu JM, Cheuk I, Ng EK, Kwong A. Circulating cell-free miRNAs as biomarker for triple-negative breast cancer. Br J Cancer. 2015;112:1751-1759.
29. Taverna S, Pucci M, Giallombardo M, et al. Amphiregulin contained in NSCLC-exosomes induces osteoclast differentiation through the activation of EGFR pathway. Sci Rep. 2017;7:3170.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.