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Original Article

Sema 3A plays a protective role in breast cancer-induced osteoclastogenesis and osteolysis

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Abstract: Bone metastasis is the most common complication in patients with solid tumors. Osteoclastogenesis and bone resorption contribute to the pathophysiology of osteoblastic bone metastasis, likewise with osteolytic bone metastasis types. Semaphorin 3A (Sema 3A) has been shown to inhibit RANKL-induced immunoreceptor tyrosine-based activation motif signaling and osteoclastic differentiation, as well as promoting osteogenesis. We previously demonstrated that Sema 3A derived from osteoblastic bone metastasis MCF-7 breast cancer cells stimulates osteoblastic differentiation. In this study, MCF-7 cells were shown to stimulate osteoclastic differentiation in vitro. The stimulation of osteoclastogenesis and bone resorption in RAW264.7 cells and primary mouse bone marrow-derived macrophages by MCF-7 cells was furthermore shown to be enhanced by Sema 3A shRNA or a Sema 3A-neutralizing antibody. The downregulation of Sema 3A expression in MCF-7 cells further promoted p-PLCγ2 expression in RAW264.7 cells. Our findings highlight the importance of Sema 3A as a bidirectional regulatory factor in osteoblastic metastasis: Sema 3A plays a protective role in breast cancer-induced osteoclastogenesis and osteolysis. Targeting Sema 3A for the treatment of osteoblastic metastasis, however, would require precise control and extensive further research.

Keywords: Cancer, bone metastasis, osteoclastogenesis, osteolysis, semaphorin 3A

Introduction

Bone metastasis is the most common complication in patients with solid tumors [1, 2]. It is estimated that more than 80% of breast cancer and 90% of prostate cancer patients in advanced stages harbor bone metastases. Bone metastasis is responsible for as much as 90% of cancer-associated mortality [3, 4] and is associated with clinical consequences including bone pain, skeletal fractures, hypercalcemia, and spinal cord compression. These severe complications are collectively referred to as skeletal-related events (SREs). Bone metastasis severely decreases the quality of life and 5-year survival rates of patients.

Based on radiographic features, approximately 15% of breast cancer cases and 90% of prostate cancer cases are osteoblastic bone metastases [5-7]. The newly formed bone is woven bone formed from collagen fibers that are randomly oriented and loosely packed, resulting in weak bone that is more susceptible to fractures [8]. The differentiation and activity of osteoblasts and osteoclasts are both enhanced in osteoblastic bone metastasis sites and cancer bone metastasis, and bone lesions have been attributed to the interaction between cancer cells and the bone marrow microenvironment in a so-called 'vicious cycle'. It is widely recognized that cancer cell-derived factors such as bone morphogenic proteins (BMPs), endothelin-1 (ET1), and platelet-derived growth factor-BB (PDGF-BB) may promote osteoblastic differentiation and new bone formation [8-10]. Antibone resorption strategies using bisphosphonate or receptor activator of NF-κB ligand (RANKL) monoclonal antibody have been shown to be effective in relieving the bone pain and delay the bone lesions resulting from bone metastasis. Considering the important role that osteoclasts play in osteoblastic metastasis, many studies have been conducted to explore the potential roles of osteoclasts and related

cellular and molecular mechanisms in osteoblastic metastasis.

Semaphorin-3A (Sema 3A), a secreted axon guidance molecule, has been shown to play a role in cancer cell migration, invasion, and angiogenesis [9, 10]. Hayashi et al recently demonstrated that Sema 3A produced by osteoblasts has bidirectional regulatory effects on osteoblasts and osteoclasts [11]: Sema 3A was found to bind to a specific receptor and stimulate osteoblastic differentiation through the canonical Wnt/β-catenin signaling pathway; while osteoclastic differentiation was also found to be inhibited by Sema 3A via the suppression of RANKL-induced immunoreceptor tyrosine-based activation motif (ITAM) signaling. It is therefore conceivable that Sema 3A produced by cancer cells may significantly influence bone rebuilding in which cancer cells, osteoblasts, and osteoclasts interact and stimulate the development of bone metastases.

We recently showed that Sema 3A plays a vital role in the osteoblastic differentiation induced by MCF-7 breast cancer cells [12], and the aim of this study was, therefore, to further explore the role of Sema 3A in the crosstalk between tumor cells and osteoclasts. We show here that Sema 3A downregulation by shRNA or a neutralizing antibody further facilitates osteoclastogenesis and bone resorption induced by MCF-7 breast cancer cells and that MCF-7 cell-derived Sema 3A stimulates Neuropilin-1 (NRP1) expression and inhibits phospholipase Cγ2 (PLCγ2) phosphorylation in osteoclast precursors. These findings highlight the importance of Sema 3A as a potential target for the treatment of osteoblastic metastasis.

Materials and methods

Cell culture

RAW264.7 and MCF-7 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin. Cell cultures were maintained at 37°C in a humid environment with 5% CO_2. To collect MCF-7 conditioned medium (CM), MCF-7 cells (2×10^6) were transferred into 75 cm^2 cell culture flasks and incubated overnight. The medium was then changed to 10 mL DMEM containing 0.5% FBS and CM was collected 24 h later, centrifuged at 1,000 g for 10 min to remove cell debris, and then stored at -80°C. Sema 3A in the CM was depleted by incubation with a Sema 3A-neutralizing antibody (Santa Cruz, Dallas, TX) or IgG as previously described [12].

Primary mouse bone marrow-derived macrophages (BMMs) were prepared as osteoclast precursors as described previously [13]. Briefly, the tibias and femurs of 4- to 6-week-old C57BL/6 mice were flushed with α-MEM, after which cells were centrifuged for 5 min at 300 g. The resulting cell pellet was resuspended in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin before being cultured for 24 h. The non-adherent cells were harvested and cultured for an additional 3 days in the presence of 20 ng/mL macrophage colony-stimulating factor (M-CSF, PeproTech, Rocky Hill, NJ). Floating cells were discarded, and the remaining adherent cells were considered BMMs.

In vitro osteoclast differentiation

BMMs were seeded in 24-well plates at a density of 4×10^4 cells/well in α-MEM supplemented with 10% FBS, M-CSF (20 ng/mL), and RANKL (50 ng/mL, PeproTech, Rocky Hill, NJ) in the presence or absence of 10% MCF-7 CM. Medium was changed every 2-3 days for 11 days. RAW264.7 cells were seeded in 24-well plates at a density of 2×10^4 cells/well in DMEM supplemented with 10% FBS and RANKL (50 ng/mL) in the presence or absence of 10% MCF-7 CM. Medium was changed every 2-3 days for 5 days.

Tartrate-resistant acid phosphatase (TRAP) staining of RAW264.7 cells and BMMs

To confirm the presence of osteoclasts, the cultured cells were stained for TRAP using a TRAP Staining Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. TRAP-positive multinucleated cells (MNCs; three or more nuclei) were considered osteoclast-like (OCL) cells and counted under light microscopy at a magnification of 50 × in five fields. Data are reported as number of TRAP-positive MNCs. Each osteoclast formation assay was performed at least three times.

Table 1. Primer sequences used for quantitative real-time-PCR

Gene	Primer sequences (5'-3')
CTR	F: GCTTGCGGTGGTATTATCTCTTGG
	R: CACTCAGCCAGCAGTTGTCATTG
TRAP	F: TGACCACCTTGGCAATGTCTCTG
	R: GATCTTGAAGTGCAGGCGGTAGAA
CK	F: GGAAGAAGACCCACAGGAAGCAATA
	R: GAGAAGCCTCAAGGTTATGGATGGA
β-actin	F: GATGTGGATCAGCAAGCAGGAGTA
	R: GCTCAGTAACAGTCCGCCTAGAAG

Bone resorption assay

Bone slices (7 × 7 mm, 50 μm thick) were cut from bovine cortical femur with a low-speed, water-cooled diamond saw (Buehler, Lake Bluff, IL). Bone slices were cleaned by ultrasonication for 2 min in distilled water and then placed in 70% ethanol overnight. Bone slices were incubated in DMEM overnight and then incubated with RAW264.7 cells (4×10^4 cells/well) in 24-well plates. RAW-264.7 cells were treated with RANKL (50 ng/mL) in the presence or absence of 10% MCF-7 CM. Medium was changed every 2-3 days for 5 days, after which the RAW264.7 cells were removed from the bone slices by sonication for 2 min in 5 mL ammonium hydroxide (0.25 M). Resorption pits were visualized under a Quanta 250 scanning electron microscope (FEI, Hillsboro, OR). Percentage resorbed bone surface area was quantified using Image J software (National Institutes of Health; http://imagej.nih.gov/ij/).

Transfection of Sema 3A shRNA

MCF-7 cells were transfected with shRNA lentiviral particles directed against Sema 3A (5'-TGCAGAAGATGGACAGTAT-3') according to manufacturer's instructions (GenePharma, Shanghai, China). This method of down-regulation was shown to substantially inhibit Sema 3A expression in our previous studies. A non-targeted oligonucleotide was used as a negative control. Stably transfected cells of both types were selected using 2.5 μg/mL puromycin (Sigma).

Western blot analysis

Cells were lysed on ice for 30 min by radioimmunoprecipitation assay buffer supplemented with 1 mM PMSF (Sigma, St. Louis, MO). Protein concentration was determined by BCA protein assay kit (Beyotime, Jiangsu, China). Cell lysate samples with equal protein content were loaded and separated on 8% (w/v) SDS-PAGE before being blotted onto PVDF membrane. After blocking with TBST containing 5% (w/v) bovine serum albumin for 1 h, the membranes were incubated with primary antibodies (NRP1 1:500; PLCγ2 1:500; phospho-PLCγ2 1:500; GAPDH 1:1000) overnight, then with horseradish peroxidase-labeled secondary antibodies (1:5000) for 1 h. The blots were visualized using an ECL chemiluminescence Kit (Bio-Rad, Cambridge, MA) and quantified using Quantity One analysis software (Bio-Rad).

Quantitative real-time-PCR

Total RNA was isolated from RAW264.7 cells using RNAiso Plus reagent (Takara, Dalian, China). Single-stranded cDNA was generated from 1 μg of total RNA using PrimeScript RT reagent kit (Takara). Subsequently, quantitative real-time PCR was performed on an ABI 7500 Sequencing Detection System (95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s) using SYBR® Premix Ex Taq™ II (Takara). The osteclastogenesis marker genes cathepsin K (CK), calcitonin receptor (CTR), and TRAP were amplified with specific primer pairs and β-actin was included as housekeeping gene. The primer sequences used for qRT-PCR are listed in **Table 1**. Relative mRNA expression levels of each gene were calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Differences between groups were evaluated by one-way analysis of variance (ANOVA) using SPSS software system (version 17.0; SPSS, Chicago, IL, USA). Differences with *P*-values < 0.05 were considered statistically significant and all date reported here were obtained from at least three independent experiments with a similar trend.

Results

MCF-7 cells stimulate osteoclastic differentiation

Before exploring the role of Sema 3A in the osteoclast differentiation induced by osteoblastic breast cancer cells, the effects of MCF-7

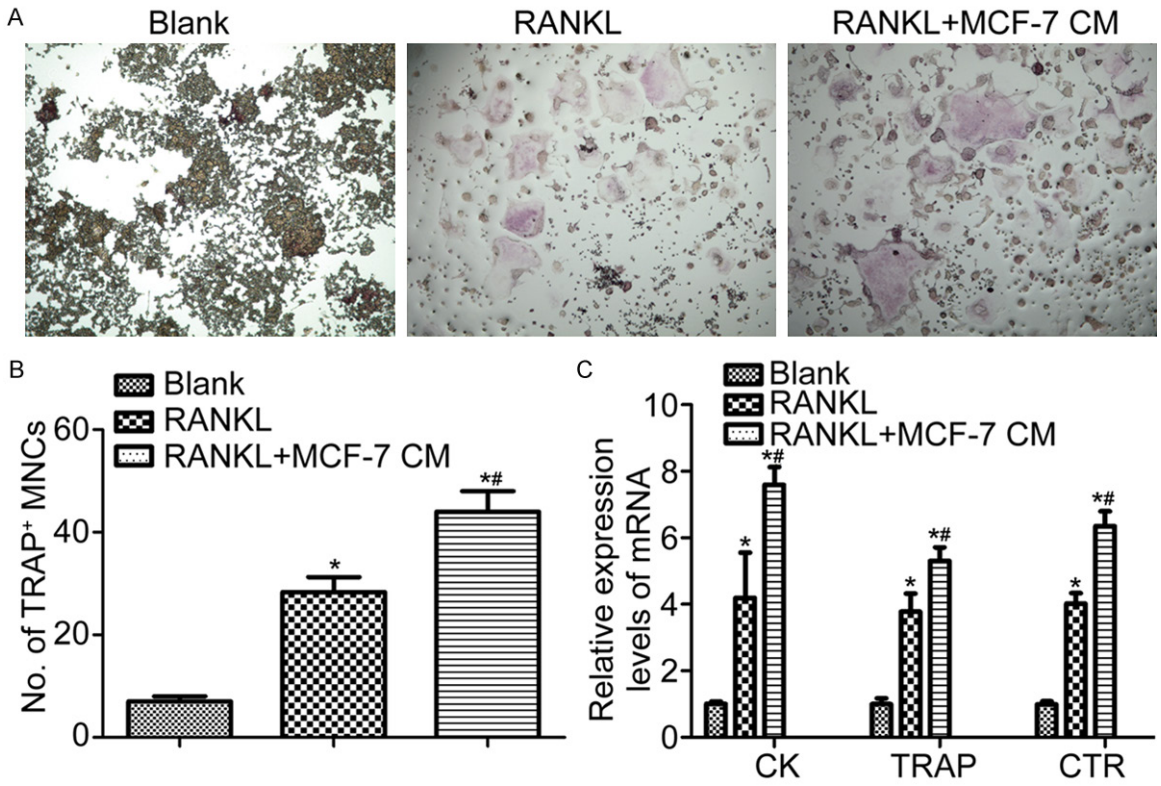

Figure 1. MCF-7 breast cancer cells augment osteoclastic differentiation. RAW264.7 cells were cultured in the absence or presence of 10% (v/v) MCF-7 cell conditioned medium (CM) for 2 hours before being treated with RANKL (50 ng/mL) for 5 days. Tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclasts. A. Representative TRAP staining images. B. Osteoclast numbers were determined from five random fields at a magnification of 50 ×. C. The relative mRNA expression levels of the osteoclast marker genes cathepsin K (CK), calcitonin receptor (CTR), and TRAP were determined by quantitative real-time PCR. *P < 0.05 vs. the Blank group; n = 3.

breast cancer cells on osteoclastic differentiation were assessed. RAW264.7 cells were cultured with RANKL in the presence or absence of MCF-7 cell-CM. After 5 days of incubation, TRAP staining revealed more TRAP-positive MNCs in the presence of MCF-7 CM (**Figure 1A** and **1B**). To further investigate the promoting effect of MCF-7 cells on osteoclastogenesis, the mRNA expression levels of osteoclastogenesis-related genes were also evaluated. CK, TRAP, and CTR are expressed during osteoclast differentiation, thereby characterizing the osteoclast phenotype. Our result showed the mRNA expression levels of CK, CTR, and TRAP to be significantly higher in cells treated with RANKL; and treatment of cells with MCF-7 CM was shown to further promote the expression of CK, TRAP, and CTR mRNA induced by RANKL (**Figure 1C**). Collectively, these findings indicate that MCF-7 cells augment osteoclastic differentiation of RAW264.7 cells.

MCF-7 cell-derived Sema 3A attenuates RAW264.7 cell osteoclastic differentiation and activity induced by MCF-7 cells

We and other researchers have reported findings that suggest that MCF-7 breast cancer cells with osteoblastic bone lesion potential exhibit high Sema 3A expression levels [12, 14]. Meanwhile, it has been demonstrated that Sema 3A suppresses osteoclastic differentiation by blocking the ITAM signaling pathways in RAW264.7 cells [11]. To study the effects of Sema 3A on osteoclastic formation in the pathological process of cancer-associated osteoblastic bone metastasis, RAW264.7 cells were cultured in the presence of RANKL for 5 days with or without MCF-7 cell CM containing different Sema 3A protein levels. Downregulation of Sema 3A expression in MCF-7 cells by shRNA was found to further enhance the number of TRAP-positive MNCs induced by MCF-7 cell CM

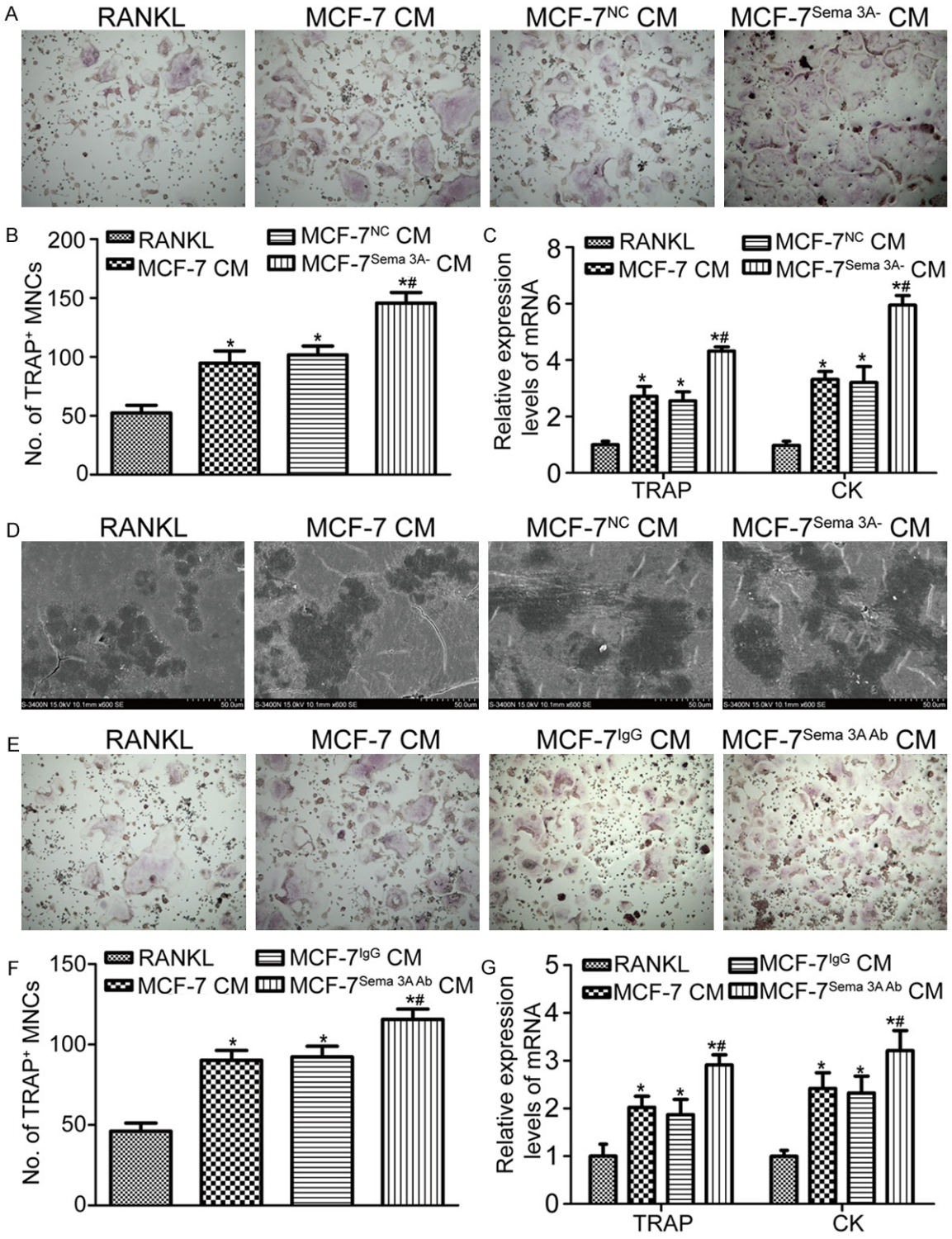

Figure 2. Reducing Sema 3A expression in MCF-7 cells further enhances the osteoclastic differentiation and bone resorption induced by MCF-7 cells in RAW264.7 cells. A-D. Sema 3A expression in MCF-7 cells was downregulated by transfection with a lentiviral vector expressing Sema 3A shRNA or a non-targeted oligonucleotide. RAW264.7 cells were then treated with MCF-7 cell CM and RANKL as previously described. A. Representative TRAP staining images (50 × magnification). B. The number of TRAP-positive MNCs was determined from five randomly-selected fields. C. The mRNA expression levels of TRAP and CK were assessed by qPCR. D. Resorbed bone surfaces were visualized by scanning electron microscopy. E-G. RAW264.7 cells were cultured with MCF-7 cell CM in the presence of Sema 3A-neutralizing antibody or IgG. E. Representative TRAP staining images (50 × magnification). F. The number of TRAP-positive MNCs. G. mRNA expression levels of TRAP and CK. *$P < 0.05$ vs. the RANKL group; #$P < 0.05$ vs. the MCF-7 CM group; n = 3.

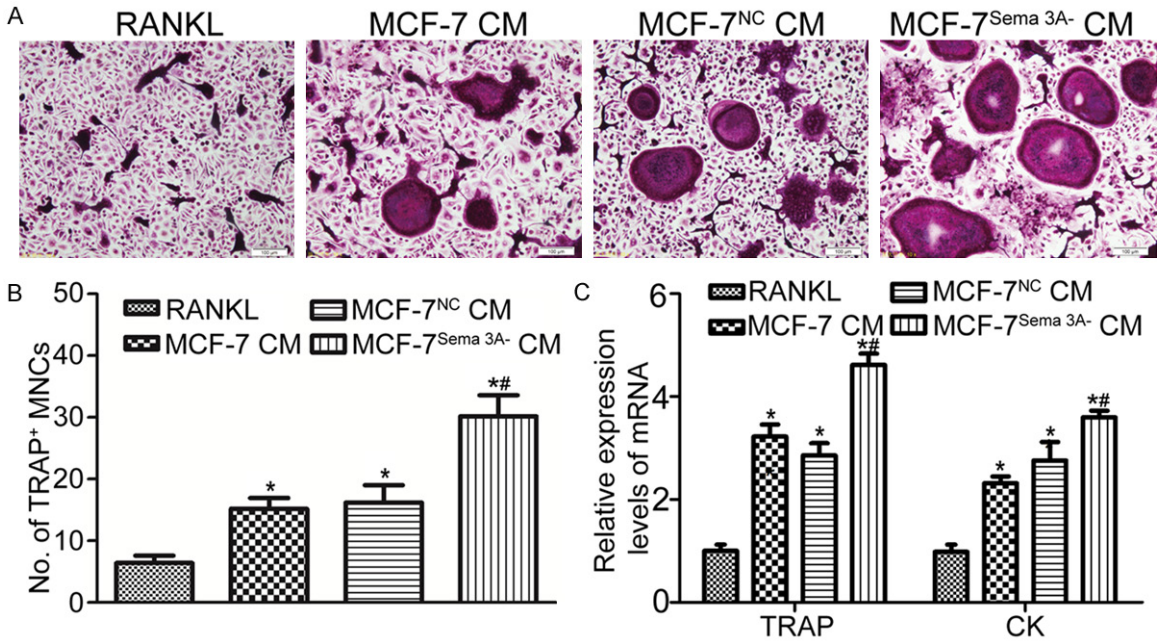

Figure 3. Reducing Sema 3A expression in MCF-7 cells further enhances bone marrow-derived macrophage (BMM) osteoclastic differentiation induced by MCF-7 cells. Sema 3A expression in MCF-7 cells was downregulated by an shRNA lentiviral vector, after which BMM cells were treated with MCF-7 cell CM and RANKL. A. Representative TRAP staining images (50 × magnification). B. The number of TRAP-positive MNCs was determined from five randomly-selected fields. C. The mRNA expression levels of TRAP and CK were assessed by qPCR. *$P < 0.05$ vs. the RANKL group; #$P < 0.05$ vs. the MCF-7 CM group; $n = 3$.

(**Figure 2A** and **2B**). The mRNA expression levels of the osteoclast marker genes TRAP and CK in RAW264.7 cells was assessed by qPCR, and as expected, the mRNA expression of TRAP and CK was further upregulated in response to MCF-7$^{Sema\ 3A-}$ CM (**Figure 2C**). To further determine the effect of Sema 3A on osteoclast function (bone resorption) induced by MCF-7 CM, bovine bone slices were incubated with RAW264.7 cells in the absence or presence of MCF-7 CM. Scanning electron microscopy (**Figure 2D**) revealed that the bone resorption area of RAW264.7 cells treated with MCF-7 CM was double the size of that induced by RANKL alone. Unexpectedly, the bone resorption area in MCF-7$^{Sema\ 3A-}$ CM group was further significantly promoted compared to MCF-7 CM group. More importantly, the effects of the MCF-7 CM on TRAP staining and the expression of osteoclast marker genes were also reduced by the Sema 3A-neutralizing antibody but not by non-specific polyclonal goat IgG (**Figure 2E-G**). Taken together, these findings clearly demonstrate that Sema 3A attenuated the osteoclastic differentiation and activity induced by MCF-7 cells in RAW264.7 cells.

MCF-7 cell-derived Sema 3A attenuates osteoclastic differentiation in BMMs induced by MCF-7 cells

To further determine the effects of Sema 3A derived from MCF-7 cells on osteoclasts, BMMs were also treated with MCF-7 cell CM in the presence of M-CSF and RANKL. After incubation for 11 days, the BMMs were stained for TRAP and the TRAP-positive MNCs were counted. The results showed that MCF-7 cell CM also markedly increased the number of TRAP-positive MNCs relative to the number in the group treated with RANKL alone. Meanwhile, downregulating Sema 3A expression in MCF-7 cells also further promoted BMM osteoclastic differentiation induced by MCF-7 cell CM (**Figure 3A** and **3B**). The mRNA expression levels of TRAP and CK in BMMs were also further upregulated in response to Sema 3A shRNA (**Figure 3C**).

Sema 3A stimulates NRP1 expression but inhibits PLCγ2 phosphorylation in RAW264.7 cells

NRP1 is the exclusive Sema 3A receptor that supports Sema 3A signaling [15, 16]. Sema 3A

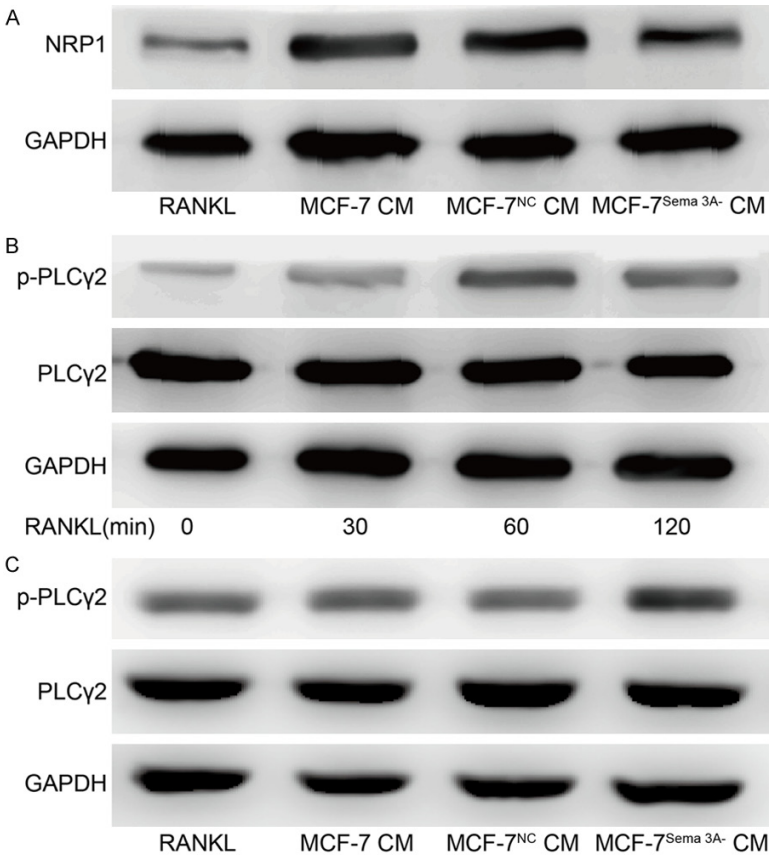

Figure 4. MCF-7 cell-derived Sema 3A stimulates NRP1 expression but inhibits PLCγ2 phosphorylation in RAW264.7 cells. (A) RAW264.7 cells were treated with MCF-7 cell CM before being treated with RANKL for 1 day. NRP1 expression in RAW264.7 cells was examined as previously described. (B) RAW264.7 cells were treated with RANKL for indicated times and the expression levels of p-PLCγ2 and PLCγ2 were determined with specific antibodies. (C) RAW264.7 cells were treated with MCF-7 cell CM, incubated with RANKL for 60 min, and then analyzed for p-PLCγ2 and PLCγ2 expression (B).

treatment interferes with RANKL-induced Plexin-A1-TREM2-DAP12 complex formation by inhibiting NRP1 downregulation, subsequently blocking tyrosine phosphorylation of PLCγ2, and finally inhibiting the osteoclastic differentiation in osteoclast precursors [11]. To assess the importance of NRP1 for Sema 3A regulation of osteoclastic differentiation in bone metastasis, NRP1 protein expression in RAW264.7 cells stimulated with RANKL in the presence or absence of MCF-7 cell CM for 1 day was assessed by western blot analysis. The protein expression of NRP1 was found to be highly upregulated in the MCF-7 cell CM treatment group, whereas this stimulation of NRP1 expression was markedly attenuated in RAW264.7 cells treated with MCF-7$^{Sema\ 3A-}$ CM (**Figure 4A**).

Next we investigated whether PLCγ2 activity is involved in osteoclastic differentiation induced by MCF-7 cell CM. RAW264.7 cells were cultured with RANKL for different durations before being analyzed for phosphorylated PLCγ2 (p-PLCγ2) levels. As shown in **Figure 4B**, p-PLCγ2 levels were found to increase in a time-dependent manner and peaked with 60 min incubation, whereas PLCγ2 expression did not vary significantly with time. The addition of MCF-7 cell CM was also shown to have no significant effect on p-PLCγ2 levels. By contrast, p-PLCγ2 expression was shown to be promoted by the downregulation of Sema 3A in MCF-7 cells (**Figure 4C**).

Discussion

Advances in oncological research have resulted in the overall life expectancy of cancer patients improving in recent years, and accordingly, the number of patients suffering from cancer-induced bone lesions has increased. There are, however, few successful treatment options for patients with bone metastasis that improve overall patient survival. Gaining a greater understanding of the cellular and molecular mechanisms involved in bone metastasis may facilitate the design of more effective and specific treatments for cancer patients with bone metastases. In the present study, we examined the role of Sema 3A in regulating the bone rebuilding induced by MCF-7 human breast cancer cells. Sema 3A downregulation by shRNA or a neutralizing antibody was shown to further facilitate osteoclastogenesis and bone resorption induced by MCF-7 breast cancer cells. These findings indicate that targeting Sema 3A may provide a novel therapeutic approach for the treatment of metastatic breast cancer.

Osteoclastogenesis and bone resorption also contribute to the pathophysiology of bone osteoblastic metastasis, likewise with osteolytic metastasis types. Osteoclastic bone resorption may be essential for osteoblastic metastasis to initiate new bone formation [17]. Although often characterized as osteoblastic, bone metastases in most prostate cancer patients usually have an underlying osteolytic component in conjunction with osteosclerosis [18, 19]. Osteoclast formation induced by macrophage inhibitory cytokine-1 has been shown to significantly promote the development of prostate cancer in a mouse model [20]. Meanwhile, it must be noted that osteoblastic bone metastasis occurs in a significant number of breast cancer patients, although osteolytic metastasis is the most common form of the metastasis [8]. MCF-7 is a typical and commonly used breast cancer cell line, which preferentially forms osteoblastic bone metastasis [21, 22]. In an MCF-7 xenograft mouse model, osteoclast number and activity were both found to be markedly elevated, resulting in the appearance of radiographically evident bone lesions [22].

Bisphosphonates are the drugs currently available for the treatment of osteoblastic metastasis [23]; however, even the most powerful of these agents, zoledronic acid, only prevents a portion of SREs [24]. Meanwhile, it has been reported that bisphosphonate may promote osteoblastic differentiation, which may be harmful for osteoblastic metastasis [25]. RANKL inhibition has revolutionized the management of various bone metastases, representing the spectrum of osteolytic, osteoblastic, or mixed lesion types, reducing both bone destruction and skeletal tumor progression [26, 27]. The high cost and severe side effects associated these two therapies, including renal impairment and jaw osteonecrosis, require careful consideration when deciding on a treatment strategy for bone metastasis [24]. While the pursuit for safe and effective therapies for osteoblastic metastasis continues, the cellular and molecular mechanisms underlying cancer osteoblastic bone lesion have not been fully elucidated.

Sema 3A, a secreted member of the semaphorin family, has been shown to have dual-regulatory effects on normal bone rebuilding by controlling the differentiation and activity of osteoblasts and osteoclasts [11]. Bone homeostasis depends on the balance between osteoblast-induced bone formation and osteoclast-induced bone resorption during bone remodeling. The role of Sema 3A has been explored in many bone-related pathologic statuses including osteoporosis [28, 29] and lower back pain [30]. Sema 3A exerts its biological activity via interactions with NRP1. Sema 3A facilitates the translocation of β-catenin, thereby stimulating osteoblastic differentiation. The binding of Sema 3A to NRP1 on the other hand also sequesters Plexin-A1 from TREM2-DAP12, which is responsible for RANKL-induced ITAM signaling and osteoclastic differentiation. We previously reported that Sema 3A expression is far higher in osteoblastic MCF-7 cells than in MDA-MB-231 breast cancer osteolytic cells, which is consistent with previous studies [12, 14]. This observation suggests that Sema 3A derived from breast cancer cells may play a role in osteoclastic differentiation and bone resorption in osteoblastic metastasis.

In this study, MCF-7 CM was found to facilitate the osteoclastic differentiation of RAW264.7 cells and BMMs, which is in agreement with previously reported findings [31, 32]. Interestingly, downregulating Sema 3A expression in MCF-7 cells resulted in an enhancement of this MCF-7 CM effect. An assessment of resorption pits in bone slices revealed that bone resorption activity was likewise further promoted by shRNA-mediated Sema 3A downregulation in MCF-7 cells. To assess the nature of the regulatory effect of the Sema 3A produced by MCF-7 cells on osteoclastic differentiation, the activity of PLCγ2, a downstream ITAM signaling molecule, was assessed. p-PLCγ2 expression was shown to be markedly stimulated by RANKL. A clear increase in p-PLCγ2 levels after the addition of MCF-7$^{Sema\ 3A-}$ CM (CM from cells in which Sema 3A expression was inhibited by shRNA) was observed. Taken together, these findings suggest that Sema 3A derived from MCF-7 cells may act as a permissive factor for osteoblastic bone metastasis of MCF-7 breast cancer cells by inhibiting osteoclastic differentiation, although other factors produced by MCF-7 cells may promote osteoclastic differentiation. Given that MCF-7 cell-derived Sema 3A stimulates osteoblastic MC3T3-E1 cell differentiation, Sema 3A may serve as an important

target for the treatment for osteoblastic metastasis. It should be noted, however, that this study was only conducted in an in vitro model, and that further studies are therefore required to determine whether Sema 3A has the same effects in osteoblastic metastases animals and humans.

It should also be noted that osteoclastic bone resorption may contribute to the pathophysiology of osteoblastic metastasis. Bisphosphonate has been shown to reduce skeletal morbidity in patients with osteoblastic disease [33]. RANKL inhibition by osteoprotegerin-Fc treatment was found to block osteoclast activity and prevent tumor-induced osteolysis as well as to markedly decrease the skeletal tumor burden in a breast cancer MCF-7 cell bone lesion model [22]. Research published in this field has indicated that the inhibition of osteoclastic differentiation and activity may decrease the morbidity of SREs and step down the process of osteoblastic metastasis. In this way, cancer cell-derived Sema 3A may play a protective role for cancer osteoblastic lesions, while also inhibiting the excessive osteoclastic differentiation induced by cancer cells. Inhibiting the expression of Sema 3A or neutralizing its function in the microenvironment of bones with metastasis is not an effective strategy for the treatment of osteoblastic metastasis. In fact, Sema 3A can be considered a potentially effective therapeutic target for osteoblastic metastasis only when osteoblast enhancement by Sema 3A inhibition can be achieved without impacting its effect on osteoclasts (and/or cancer cells).

In conclusion, the findings reported here reveal novel mechanisms for osteoblastic metastasis. Besides the promotion of osteoblastic differentiation, cancer cell-derived Sema 3A was shown to block osteoclastogenesis and bone resorption, consequently regulating cancer osteoblastic bone lesions. Although the work presented here indicates that Sema 3A plays an important role in the cancer bone microenvironment, our study was restricted to in vitro cell experiments. Further research is required to determine whether the experimental findings described here can be extrapolated to osteoblastic bone metastases in breast cancer and prostate cancer patients.

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Disclosure of conflict of interest

None.

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References

[1] Berman AT, Thukral AD, Hwang WT, Solin LJ and Vapiwala N. Incidence and patterns of distant metastases for patients with early-stage breast cancer after breast conservation treatment. Clin Breast Cancer 2013; 13: 88-94.

[2] Suva LJ, Washam C, Nicholas RW and Griffin RJ. Bone metastasis: mechanisms and therapeutic opportunities. Nat Rev Endocrinol 2011; 7: 208-218.

[3] Chaffer CL and Weinberg RA. A perspective on cancer cell metastasis. Science 2011; 331: 1559-1564.

[4] AACR Cancer Progress Report Writing Committee, Sawyers CL, Abate-Shen C, Anderson KC, Barker A, Baselga J, Berger NA, Foti M, Jemal A, Lawrence TS, Li CI, Mardis ER, Neumann PJ, Pardoll DM, Prendergast GC, Reed JC, Weiner GJ. AACR Cancer Progress Report 2013. Clin Cancer Res 2013; 19: S4-98.

[5] Kozlow W and Guise TA. Breast cancer metastasis to bone: mechanisms of osteolysis and implications for therapy. J Mammary Gland Biol Neoplasia 2005; 10: 169-180.

[6] Larson SR, Zhang X, Dumpit R, Coleman I, Lakely B, Roudier M, Higano CS, True LD, Lange PH, Montgomery B, Corey E, Nelson PS, Vessella RL and Morrissey C. Characterization of osteoblastic and osteolytic proteins in prostate cancer bone metastases. Prostate 2013; 73: 932-940.

[7] Lu Y, Cai Z, Xiao G, Keller ET, Mizokami A, Yao Z, Roodman GD and Zhang J. Monocyte chemotactic protein-1 mediates prostate cancer-induced bone resorption. Cancer Res 2007; 67: 3646-3653.

[8] Mohammad KS and Guise TA. Mechanisms of osteoblastic metastases: role of endothelin-1. Clin Orthop Relat Res 2003; S67-74.

[9] Gaur P, Bielenberg DR, Samuel S, Bose D, Zhou Y, Gray MJ, Dallas NA, Fan F, Xia L, Lu J

and Ellis LM. Role of class 3 semaphorins and their receptors in tumor growth and angiogenesis. Clin Cancer Res 2009; 15: 6763-6770.
[10] Neufeld G, Sabag AD, Rabinovicz N and Kessler O. Semaphorins in angiogenesis and tumor progression. Cold Spring Harb Perspect Med 2012; 2: a006718.
[11] Hayashi M, Nakashima T, Taniguchi M, Kodama T, Kumanogoh A and Takayanagi H. Osteoprotection by semaphorin 3A. Nature 2012; 485: 69-74.
[12] Shen WW, Chen WG, Liu FZ, Hu X, Wang HK, Zhang Y and Chu TW. Breast cancer cells promote osteoblastic differentiation via Sema 3A signaling pathway in vitro. Int J Clin Exp Pathol 2015; 8: 1584-1593.
[13] Wang H, Shen W, Hu X, Zhang Y, Zhuo Y, Li T, Mei F, Li X, Xiao L and Chu T. Quetiapine inhibits osteoclastogenesis and prevents human breast cancer-induced bone loss through suppression of the RANKL-mediated MAPK and NF-kappaB signaling pathways. Breast Cancer Res Treat 2015; 149: 705-714.
[14] Bachelder RE, Lipscomb EA, Lin X, Wendt MA, Chadborn NH, Eickholt BJ and Mercurio AM. Competing autocrine pathways involving alternative neuropilin-1 ligands regulate chemotaxis of carcinoma cells. Cancer Res 2003; 63: 5230-5233.
[15] Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ and Ginty DD. Neuropilin is a semaphorin III receptor. Cell 1997; 90: 753-762.
[16] He Z and Tessier-Lavigne M. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 1997; 90: 739-751.
[17] Zhang J, Dai J, Qi Y, Lin DL, Smith P, Strayhorn C, Mizokami A, Fu Z, Westman J and Keller ET. Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. J Clin Invest 2001; 107: 1235-1244.
[18] Keller ET. The role of osteoclastic activity in prostate cancer skeletal metastases. Drugs Today (Barc) 2002; 38: 91-102.
[19] Scher HI and Chung LW. Bone metastases: improving the therapeutic index. Semin Oncol 1994; 21: 630-656.
[20] Wakchoure S, Swain TM, Hentunen TA, Bauskin AR, Brown DA, Breit SN, Vuopala KS, Harris KW and Selander KS. Expression of macrophage inhibitory cytokine-1 in prostate cancer bone metastases induces osteoclast activation and weight loss. Prostate 2009; 69: 652-661.
[21] Gupta J, Robbins J, Jilling T and Seth P. TGFbeta-dependent induction of interleukin-11 and interleukin-8 involves SMAD and p38 MAPK pathways in breast tumor models with varied bone metastases potential. Cancer Biol Ther 2011; 11: 311-316.
[22] Canon J, Bryant R, Roudier M, Branstetter DG and Dougall WC. RANKL inhibition combined with tamoxifen treatment increases anti-tumor efficacy and prevents tumor-induced bone destruction in an estrogen receptor-positive breast cancer bone metastasis model. Breast Cancer Res Treat 2012; 135: 771-780.
[23] Yonou H, Ochiai A, Ashimine S, Maeda H, Horiguchi Y, Yoshioka K, Ogawa Y, Hatano T and Tachibana M. The bisphosphonate YM529 inhibits osteoblastic bone tumor proliferation of prostate cancer. Prostate 2007; 67: 999-1009.
[24] Brown JE and Coleman RE. Denosumab in patients with cancer-a surgical strike against the osteoclast. Nat Rev Clin Oncol 2012; 9: 110-118.
[25] Reinholz GG, Getz B, Sanders ES, Karpeisky MY, Padyukova N, Mikhailov SN, Ingle JN and Spelsberg TC. Distinct mechanisms of bisphosphonate action between osteoblasts and breast cancer cells: identity of a potent new bisphosphonate analogue. Breast Cancer Res Treat 2002; 71: 257-268.
[26] Whang PG, Schwarz EM, Gamradt SC, Dougall WC and Lieberman JR. The effects of RANK blockade and osteoclast depletion in a model of pure osteoblastic prostate cancer metastasis in bone. J Orthop Res 2005; 23: 1475-1483.
[27] Miller RE, Roudier M, Jones J, Armstrong A, Canon J and Dougall WC. RANK ligand inhibition plus docetaxel improves survival and reduces tumor burden in a murine model of prostate cancer bone metastasis. Mol Cancer Ther 2008; 7: 2160-2169.
[28] Kang S and Kumanogoh A. Semaphorins in bone development, homeostasis, and disease. Semin Cell Dev Biol 2013; 24: 163-171.
[29] Tang P, Yin P, Lv H, Zhang L and Zhang L. The Role Of Semaphorin 3A In The Skeletal System. Crit Rev Eukaryot Gene Expr 2015; 25: 47-57.
[30] Tolofari SK, Richardson SM, Freemont AJ and Hoyland JA. Expression of semaphorin 3A and its receptors in the human intervertebral disc: potential role in regulating neural ingrowth in the degenerate intervertebral disc. Arthritis Res Ther 2010; 12: R1.
[31] Nicolin V, Bortul R, Bareggi R, Baldini G, Martinelli B and Narducci P. Breast adenocarcinoma MCF-7 cell line induces spontaneous osteoclastogenesis via a RANK-ligand-dependent pathway. Acta Histochem 2008; 110: 388-396.
[32] Lau YS, Danks L, Sun SG, Fox S, Sabokbar A, Harris A and Athanasou NA. RANKL-dependent and RANKL-independent mechanisms of macrophage-osteoclast differentiation in breast

cancer. Breast Cancer Res Treat 2007; 105: 7-16.
[33] Guise TA, Mohammad KS, Clines G, Stebbins EG, Wong DH, Higgins LS, Vessella R, Corey E, Padalecki S, Suva L and Chirgwin JM. Basic mechanisms responsible for osteolytic and osteoblastic bone metastases. Clin Cancer Res 2006; 12: 6213s-6216s.