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Lipid Osteoclastokines Regulate Breast Cancer Bone Metastasis

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Introduction

Bone metastasis is a frequent, debilitating and essentially incurable cancer complication that accounts for substantial cancer morbidity and mortality. Yet, the regulation of this complex process remains poorly understood. A bevy of tumors exhibit a strong tendency to metastasize to the bone, including breast, prostate, lung, skin, colon, stomach, bladder, uterus, rectum, thyroid and kidney cancers (1). Bone metastasis occurs in up to 70% of patients with advanced breast or prostate cancer (2), and in 15-30% of patients with many other types of cancer. It is estimated that in the United States alone at least 350,000 people die with bone metastases annually (3). Once tumors metastasize to bone, the patients are usually incurable: only 20% of women with breast cancer are still alive 5 years after the discovery of bone metastasis (4). The consequences of bone metastases are often devastating: severe bone pain, pathologic fractures, life-threatening hypercalcemia, anemia, spinal cord compression, limited mobility, impaired quality of life and many others. For all these reasons, it is of paramount urgency and importance to develop new cancer remedies that target bone metastasis, which requires further understanding of mechanisms controlling both the tumor cells and the bone metastatic niche.

In breast cancer bone metastasis particularly, a vicious cycle has been recognized in the osseous environment, whereby bi-directional interactions between tumor cells and osteoclasts lead to both bone loss and tumor growth (5-8). It has been proposed that tumor cells produce factors that directly or indirectly induce osteoclastogenesis; in turn, excessive bone resorption by osteoclasts not only causes bone destruction but also releases growth factors from the bone matrix that stimulate tumor seeding and proliferation (5-8). Indeed, clinical application of
osteoclast inhibitors, such as bisphosphonates and RANKL neutralizing antibody (denosumab), can interrupt this vicious cycle, thereby reducing bone lesions and tumor burden (3,9,10).

Our recent discoveries of novel regulators of osteoclastogenesis and establishment of new mouse genetic models have paved the road to further elucidate the roles of osteoclast in cancer bone metastasis. We have identified the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARG) as a pro-osteoclastogenic transcription factor (11), and osteoclastic PPARγ knockout mice (Oc-PPARg-KO) exhibit osteopetrosis with decreased osteoclasts and bone resorption (11). We have also identified β-catenin as a key biphasic and dosage-dependent regulator of osteoclastogenesis (12). Our mouse genetic models show that osteoclast differentiation and bone resorption is impaired by osteoclastic β-catenin constitutive activation (Oc-bCat-CA) or osteoclastic β-catenin deletion (Oc-bCat-KO), but enhanced by osteoclastic β-catenin heterozygosity (Oc-bCat-Het) (12). This finding was confirmed by subsequent studies (13). Moreover, we have uncovered the microRNA miR-34a as a powerful suppressor of osteoclastogenesis, bone resorption and cancer bone metastasis, by using osteoclastic miR-34a transgenic and knockout mouse genetic models as well as nanoparticle miR-34a delivery mouse pharmacological models (15).

In this study, we have found that osteoclast also exhibits an ability to directly stimulate breast cancer bone metastasis by reprogramming osteoclast cytokines (osteoclastokines), and identified a critical paracrine-signaling pathway in which osteoclast-secreted lipid factors promote tumor cell proliferation, migration, survival, and expression of pro-metastatic genes. These findings uncover new mechanisms underlying the tumor-osteoclast vicious cycle, and reveal lipids as key mediators and therapeutic targets of cancer bone metastasis.

Materials and methods

Mice
As we previously described (11), osteoclastic PPARγ knockout (Oc-PPARg-KO) mice driven by Tie2Cre exhibit impaired osteoclastogenesis. As we previously described (12), osteoclastic β-catenin constitutive active (Oc-bCAl) mice driven by PPARγ-tTA; TRE-cre (PT-Cre) (14) or Lysozyme-Cre (Ly-Cre) exhibit similarly blunted osteoclastogenesis; osteoclastic β-catenin knockout (Oc-bKO) mice and osteoclastic β-catenin heterozygous (Oc-bHet) mice driven by PT-Cre exhibit decreased and increased osteoclastogenesis, respectively. These mice were bred with nude (athymic, nu/nu) mice to generate immunodeficient mutant and control mice for human breast cancer cell xenograft. All experiments were conducted using littermates. Sample size estimate was based on power analyses performed using SAS 9.3 TS X64_7PRO platform. All protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee of UTSW.

Reagents and Cell Lines
Arachidonic acid was from Sigma. Lysophosphatidylcholine 18:0 and 16:0 were from Avanti Polar Lipids. BW-755C was from Cayman Chemicals. Luciferase-labelled MDA-MB-231 parental line and BoM-1833 bone-metastasis-prone subline of human breast cancer cells were described (15). Luciferase-labelled Py8119 bone-metastatic-prone mouse mammary tumor cell line was originally derived from spontaneous mammary tumors in C57BL/6 MMTV-PyMT female transgenic mice (16). RAW264.7 mouse macrophage cell line was from ATCC (TIB-71).

Ex Vivo Osteoclast Differentiation and Conditioned Medium Collection
Osteoclasts were differentiated from mouse bone marrow cells as previously described (15). Briefly, bone marrow cells were purified with a 40μm cell strainer to remove mesenchymal cells.
Osteoclast precursors were expanded with 20ng/ml M-CSF for 3 days, which were then differentiated with 20ng/ml MCSF + 100ng/ml RANKL for 6 days to derive mature osteoclasts. RAW264.7 mouse macrophage cells were differentiated with 100ng/ml RANKL for 4 days to derive mature osteoclasts. On the last day of differentiation, culture medium was removed and fresh serum-free α-MEM without MCSF or RANKL was added, which was collected 24 hrs later as osteoclast conditioned medium. Control conditioned medium was collected in a similar fashion from osteoclast precursors without RANKL stimulation. The conditioned medium was filtered through 0.45μm filter, aliquoted and stored at -20°C until use. For proteinase K treatment, proteinase K was added to the conditioned medium at a final concentration of 200μg/ml, and the tubes were incubated at 55°C for 2hrs.

**Lipid Extraction and Analyses**

Collected conditioned medium was mixed with chloroform and methanol at 5:2:1 ratio in a glass vial. The sample was centrifuged for 25 min at 2,000 g and the bottom organic phase was retrieved as lipid fraction, which was dried under a stream of nitrogen and then stored at −80°C. Metabolomic profiling using untargeted LC-MS and lipid quantification using targeted LC-MS were performed as previously described (17,18). To determine in vivo osteoclast regulation of circulating AA and LPCs, 8-week-old mice were treated via i.p. injections with RANKL (R&D Systems, 20μg/mouse once per week for 4 weeks), zoledronate (Sigma, 20μg/mouse once per week for 2 weeks), or PBS control. Serum levels of AA and total LPCs were quantified using ELISA (MyBioSource).

**Cancer Cell Analyses**

To measure cancer cell proliferation, luciferase-labelled cancer cells were seeded in 96-well plates at a density of 2x10^3 cells/well in DMEM containing 10% FBS. After cells attached, culture medium was replaced with either 200μl/well CM+10% FBS, or with 200μl/well fresh DMEM+10% FBS that was supplemented with either 10μg/well purified Oc/control lipids or AA/LPC at indicated concentration. All medium was refreshed every 2 days. Cells were lysed at the indicated time points. Luciferase output from the cell lysate was quantified to measure cancer cell numbers. MTT assay was used as an alternative method to quantify cancer cell proliferation. To measure cancer cell apoptosis, cancer cells were cultured in CM without FBS supplement or serum-free-medium with lipids for 48 hrs, serum starvation-induced apoptosis was then quantified by Annexin V: PE Apoptosis Detection Kit I (BD Biosciences) following by flow cytometry. Cancer cell migration was quantified using a trans-well assay. Cells and chambers were prepared following the Cell Migration Protocol from Corning. Briefly, 5x10^5 cells were seeded in the upper chamber and 600μl CM+10% FBS or culture medium supplemented with lipids were placed in the bottom chamber. After 48 hrs, cells migrated to the bottom chamber were counted to calculate the percentage of migrating cells. Scratch wound healing assay was used as an alternative method to assess cell migration.

**Gene Expression Analysis**

Cancer cells were seeded in 12-well plate and cultured in 2ml CM+10%FBS or culture medium supplemented with the indicated lipids for 3 days before harvest. Expression of pro-metastatic genes was quantified using real-time RT-qPCR. RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s protocol. RNA was first treated with RNase-free DNase I using the DNA-free kit (Ambion) to remove all genomic DNA, and then reverse-transcribed into cDNA using an ABI High Capacity cDNA RT Kit (Invitrogen). The cDNA was analyzed using real-time quantitative PCR (SYBR Green, Invitrogen) with an Applied Biosystems 7900
Sequence Detection System. Each reaction was performed in triplicate in a 384-well format. The expression of mouse gene was normalized by mouse L19. The expression of human gene was normalized with human GAPDH.

**3D Bone Metastasis model**

In a 3D bone metastasis cell culture model, 5×10^5 luciferase-labelled cancer cells were co-cultured with a piece of calvarial bone in a roller tube, in the presence of 3ml low oxygen (mixture of 5% O_2, 5% CO_2 and 90% N_2) infused Oc CM or control CM. The tubes were sealed and placed horizontally on a rotator that rotated at 5 revolutions / min in 37°C incubator. The medium was changed and flushed with low oxygen gas every 2 days. After 7 day incubation, the calvarial bone was carefully washed and homogenized in PBS buffer, bone-residing cancer cells were quantified by luciferase readout.

**Bone Metastasis Analyses**

Bone metastasis in vivo was induced using a human breast cancer xenograft model or a mouse mammary tumor allograft model as previously described (15,19). Using a VisualSonics Vevo770 small animal ultrasound device, luciferase-labeled cancer cells were injected into the left cardiac ventricle so that they can bypass the lung and efficiently migrate to the bone. Bone metastases were detected and quantified weekly post injection by bioluminescence imaging (BLI) using a Caliper Xenogen Spectrum instrument at UTSW small animal imaging core facility. The luciferase-labeled bone-metastasis-prone MDA-MB-231 human breast cancer cell sub-line (BoM-1833) was generously provided by Joan Massagué (HHMI and Memorial Sloan-Kettering Cancer Center) and Yibin Kang (Princeton University) (19,20), and injected into 6-week-old female nude mice (NCI-Charles River) at 1×10^5 cells/mouse in 100μl PBS. The luciferase-labeled C57BL/6-compatible MMTV-PyMT mice-derived metastasis-prone mouse mammary tumor cell line Py8119 was generously provided by Jean Jiang (University of Texas Health Science Center) (21) and injected into 8-week-old female C57BL/6J mice at 2×10^4/mouse in 100μl PBS. For in vivo treatment with Oc Lipids or Ctrl Lipids, 50μg of lipids in 5μl DMSO + 95μl PBS was intravenously injected into each mouse twice a week, starting one week before Py8119 cancer cell injection and continued for two weeks after. Bone metastases were quantified by BLI weekly, and the results for two weeks post cancer cell injection are shown.

**Statistical Analyses**

All statistical analyses were performed with Student's t-Test unless noted as ANOVA or log-rank (Mantel-Cox) test. Results are shown as mean ± standard deviation (SD). The p values were designated as: *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001; n.s. non-significant (p>0.05).

**Results**

**Inhibition of osteoclastogenesis suppresses bone metastasis of cancer**

To determine how osteoclast alterations regulate breast cancer bone metastasis, we first examined our previously described osteoclastic β-catenin mouse genetic models (12). As shown in Fig. 1A, E, H, bone metastasis from human breast cancer cells developed at 80-90% penetrance in wild-type (WT) mice 5 weeks post xenograft. In contrast, bone metastasis was significantly dampened in Oc-bCat-CA (Fig. 1A-G) and Oc-bCat-KO mice (Fig. 1H-J) that had fewer osteoclasts, but exacerbated in Oc-bCat-Het mice (Fig. 1H-J) that had more osteoclasts. Reduced susceptibility of bone metastasis was also observed in our other mouse models with decreased osteoclastogenesis, including Oc-PPARg-KO mice (11) (Fig. 1F-H) and osteoclastic miR-34a transgenic mice (15); whereas augmented susceptibility of bone metastasis was
observed in our other mouse models with increased osteoclastogenesis such as miR-34a knockout mice (15). These observations using a variety of osteoporotic and osteopetrotic mouse models fully support the crucial bone-metastasis promoting role of osteoclast in vivo.

**Osteoclast-secreted factors promote tumor cell metastatic features**

We asked the question whether osteoclasts directly stimulate cancer cell bone metastasis by providing a hospital tumor microenvironment, in addition to the previously described indirect mechanism via growth factor release from the bone matrix. To analyze the direct effects of osteoclasts on cancer cells, we established an in vitro system to co-culture the BoM-1833 cancer cells with conditioned medium (CM) collected from mature bone marrow-derived osteoclasts. Compared to CM collected from vehicle-treated control cultures with no osteoclast formation, CM from RANKL-induced WT osteoclast differentiation cultures significantly increased cancer cell growth, quantified by luciferase output (Fig. 2A). This effect was largely abolished for the CM collected from bCA cultures that failed to form any osteoclasts (12) (Fig. 2A). Consistent with these observations in primary cell cultures, CM collected from osteoclasts differentiated from the RAW267.4 mouse macrophage cell line also enhanced BoM-1833 breast cancer cell growth compared to CM collected from undifferentiated control RAW cells (Fig. 2B). Moreover, osteoclast CM also accelerated cancer cell migration (Fig. 2C) and reduced cancer cell apoptosis (Fig. 2D).

To further investigate how osteoclast CM affected cancer cell seeding and proliferation on bone, we established an ex vivo 3D cancer bone metastasis model originally developed by Curtin et al (22). BoM-1833 breast cancer cells were cultured with osteoclast CM or control CM, in the presence of a piece of mouse calvarial bone in a roller tube. After 7 days, significantly more bone-residing tumor cells were detected when cultured in osteoclast CM (Fig. 2E). These results indicate that osteoclasts secret factors (osteoclastokines) to promote cancer bone metastasis by stimulating tumor cell proliferation, migration, survival, and bone seeding.

**The pro-metastatic osteoclastokines are lipids**

To determine the functionally important components in osteoclast CM, we treated the CM with proteinase K to degrade proteins but leaving lipids and inorganic small molecules intact. The ability of osteoclast CM to increase cancer cell growth was intact after proteinase K treatment (Fig. 2F), indicating that the key factors are likely non-protein. Further experiments showed that lipids extracted from osteoclast CM exhibited similar ability to promote cancer cell growth as osteoclast CM (Fig. 2F), indicating that osteoclast-secreted lipids are mainly responsible.

Importantly, the proliferation-stimulating effect of osteoclast-secreted lipids was specific to cancer cells including both the bone-metastasis-prone BoM-1833 subline and the parental MDA-MB-231 cancer cells, but not the MCF-10a normal mammary epithelial cells (Fig. 2G). Furthermore, trans-well assay showed that osteoclast-secreted lipids also facilitated cancer cell migration equally well as osteoclast CM (Fig. 2H). Collectively, these results indicate that the metastasis-promoting activity of osteoclast secretome resides in its lipid factors.

We next examined how osteoclast CM impacts the expressions of well-established pro-metastatic genes. Osteopontin (OPN) (20,23), cyclooxygenase 2 (COX2) (24,25), integrin β3 (Itgb3) (26,27), RANKL (28), PTHrP (29), and IL-1β (30) were expressed at much higher levels in the bone-met-prone BoM-1833 subline compared to the non-metastatic MDA-MB-231 parental line (Fig. 2I). Importantly, treatment with osteoclast CM significantly enhanced the expression of these genes in both cell lines (Fig. 2I).

To verify that the effects of osteoclast CM was not limited to MDA-MB-231 and BoM-1833 human breast cancer cell lines, we conducted similar experiments using a luciferase-labelled
bone-metastasis-prone mouse mammary tumor cell line (Py8119) that was derived from spontaneous mammary tumors in C57BL/6 MMTV-PyMT female transgenic mice (16). Osteoclast-secreted lipids also significantly enhanced pro-metastatic features of Py8119 cells including increased cell proliferation (Fig. 2J) and metastatic gene expression (Fig. 2K).

Py8119 tumor cells also allowed us to examine breast cancer bone metastasis in an immunocompetent setting using C57BL/6J mice to take consideration of any potential modulation by the adaptive immune system. Compared with vehicle control, intravenous daily administration of osteoclast-secreted lipids significantly exacerbated Py8119 cell bone metastasis, demonstrated by the increased metastatic sites and BLI signal intensity (Fig. 2L-N). These findings reveal that osteoclasts promote breast cancer bone metastasis by altering its secreted lipids, thereby providing a favorable bone metastatic niche.

**Metabolomic profiling detects elevated AA secretion from osteoclasts**

To pinpoint the differences between the lipids secreted by mature osteoclasts vs. undifferentiated precursors, we performed metabolomics profiling using untargeted liquid chromatography-mass spectrometry (LC-MS) analyses as we previously described (17,18,31,32). The most significantly altered lipids under negative ionization mode was poly-unsaturated fatty acids (PUFA) including arachidonic acid (AA; C20:4) and eicosapentaenoic acid (EPA, C20:5), both were elevated by > 6-fold in Oc lipids compared with Ctrl Lipids (Fig. 3A). Subsequent targeted LC-MS analysis confirmed this observation and revealed AA as the most upregulated and abundant species (Fig. 3B). We hypothesize that elevated AA may contribute to the pro-metastatic functions of osteoclast-secreted lipids.

**AA promotes breast cancer cell migration, survival and metastatic gene expression**

To dissect the effects on cancer cell metastatic features, we performed similar experiments as for Oc CM and Oc lipids. First, trans-well assay showed that AA enhanced cancer cell migration in a dose-dependent manner (Fig. 3C). Among all the dosages tested, 10μM AA promoted cancer cell migration the most. Thus, 10μM AA was used for the subsequent experiments. Second, Annexin V-staining followed by FACS analysis revealed that AA inhibited cancer cell apoptosis (Fig. 3D). Third, real time RT-QPCR illustrated that AA also augmented the expression of pro-metastatic genes including OPN, COX-2, Itgb3, RANKL, PTHrP and IL-1β, in both MDA-MB-231 and BoM-231 cancer cells (Fig. 3E). Although cancer cell growth was not significantly affected by AA treatment (Fig. 3F-G), it was significantly reduced by the treatment of a dual inhibitor of cyclooxygenases and lipoxygenases BW-755C, for Py8119, MDA-MB-231, and BoM-1833 cells (Fig. 3G-H). BW-755C treatment also abolished the abilities of Oc lipids and AA to promote cancer cell migration (Fig. 3I-J), further supporting that BW-755C is an effectively pharmacological strategy to block AA signaling. Together, these findings reveal that osteoclasts promote cancer bone metastasis, at least in part, by increasing the abundance of AA in the bone microenvironment. Thus, either reducing AA level in the bone metastatic niche or blocking AA signaling in cancer cells may exert anti-tumor and anti-metastatic effects.

**Metabolomic profiling reveals diminished LPC secretion from osteoclasts**

Metabolomic profiling using untargeted LC-MS under positive ionization mode also detected differentially regulated lipid species. The most significantly altered class of lipids was lysophosphatidylcholine (LPC) including LPC 18:0 and LPC 16:0, which were decreased by 4-7 fold in Oc lipids compared with Ctrl lipids (Fig. 4A). Subsequent targeted LC-MS analysis confirmed this observation (Fig. 4B). We hypothesize that diminished LPC may also contribute to the pro-metastatic functions of osteoclast-secreted lipids.
**LPCs inhibit breast cancer cell growth, migration and survival**

We performed a series of experiments to determine the effects of LPC 18:0 and LPC 16:0 on breast cancer cells. First, both LPCs suppressed cancer cell migration (Fig. 4C). Second, both LPCs enhanced cancer cell apoptosis, demonstrated by the higher Annexin V staining (Fig. 4D), as well as the increased expression of pro-apoptotic genes such as Bax and the decreased expression of anti-apoptotic genes such as Bcl2 (Fig. 4E). These results suggest that increased AA and decreased LPCs from osteoclast secretome have overlapping functions to promote cancer cell migration and survival. Interestingly, although cancer cell growth was unaffected by AA treatment (Fig. 3F-H), it was significantly inhibited by both LPCs, in both 231 and BoM-1833 cells (Fig. 4F-G). Moreover, although cancer cell expression of pro-metastatic genes was enhanced by AA (Fig. 3E), it was not significantly altered by either LPC (Fig. 4H). These results suggest that increased AA and decreased LPC from osteoclast secretome also exert distinct yet complementary effects to promote cancer cell migration and survival. For all the effects observed with LPCs, LPC 18:0 showed stronger regulation than LPC 16:0 (Fig. 4C-G). Together, these findings support that increased AA and decreased LPC from osteoclast secretome have overlapping functions to promote cancer cell migration and survival. Interestingly, although cancer cell growth was unaffected by AA treatment (Fig. 3F-H), it was significantly inhibited by both LPCs, in both 231 and BoM-1833 cells (Fig. 4F-G). Moreover, although cancer cell expression of pro-metastatic genes was enhanced by AA (Fig. 3E), it was not significantly altered by either LPC (Fig. 4H). These results suggest that increased AA and decreased LPC from osteoclast secretome also exert distinct yet complementary effects to promote cancer cell migration and survival. For all the effects observed with LPCs, LPC 18:0 showed stronger regulation than LPC 16:0 (Fig. 4C-G). Together, these findings support that osteoclasts promote cancer bone metastasis also by decreasing the abundance of LPCs in the bone microenvironment. Therefore, replenishing LPCs in the bone metastatic niche may exert anti-tumor and anti-metastatic effects.

**Combination of AA blockade and LPC administration impedes cancer bone metastasis**

It is intriguing how osteoclasts polarize lipid secretion to increase AA but decrease LPCs in the bone environment to synergistically confer advantages for cancer cell metastasis. We found that AA, LPCs or BW-755C did not significantly alter osteoclast differentiation (Fig. S1), indicating that these lipids modulate bone metastasis by mainly acting on cancer cells via paracrine mechanisms rather than directly acting on osteoclasts via autocrine mechanisms.

To determine the in vivo significance of osteoclast regulation of AA and LPCs, we treated mice with recombinant RANKL or a bisphosphonate zoledronate to activate or inhibit osteoclasts, respectively, and assessed the effects on serum AA and LPCs. The results showed that osteoclast activation increased AA but decreased LPCs (Fig. 5A-C), whereas osteoclast inhibition decreased AA but increased LPCs (Fig. 5D-F). These findings further bolster the notion that osteoclast is a physiologically significant regulator of circulating AA and LPCs that is sufficient to reprogram the lipid cancer environment.

With our identification of AA as a potential pro-bone-met lipid and LPCs as potential anti-bone-met lipids, we next investigated whether the simultaneous AA signaling inhibition by BW-755C and LPCs administration could attenuate breast cancer bone metastasis in vivo. Female C57BL/6J mice were pre-treated with 2 doses of combo (BW-755C + LPC 18:0 + LPC 16:0) or vehicle placebo control before cardiac injection of Py8119 cells. The combo or vehicle treatment was continued for 3 more weeks at 2 doses per week. Remarkably, combo treatment significantly reduced both the incidence and severity of bone metastasis, demonstrated by the decreased number of metastatic sites and BLI signal intensity (Fig. 5G-I). LPC treatment alone, and to a lesser extent BW-755C treatment alone, also exhibited anti-bone-met effects (Fig. S2). These findings highlight the exciting potential of lipid-based therapies as a new strategy to alleviate cancer bone metastasis.

**Discussion**

It has been recognized for decades that breast cancer cells and osteoclasts form a “vicious cycle”, in which breast cancer cells stimulate osteoclastogenesis, and osteoclasts in turn promote cancer cell seeding and growth in bone (5-8). The molecular underpinnings for how osteoclasts support...
cancer cells described to date have been mainly attributed to the release and activation of bone-matrix-embedded growth factors as the result of bone resorption (5-8). Our present study reveals an important yet previously unrecognized additional mechanism in which osteoclasts also enhance cancer cell growth and metastatic features by reprogramming lipid secretion to create a favorable bone metastatic niche (Fig. 5J). The significance and novelty of our findings reside in the following aspects. First, we uncover that osteoclasts directly stimulate cancer cell metastatic behavior in the absence of bone matrix via the secretion of lipid osteoclastokines. Second, our global metabolomic analyses reveal that osteoclasts shift lipid secretion from their undifferentiated precursors to create an AA-rich and LPCs-poor microenvironment. Third, we identify AA as a pro-metastatic lipid and LPCs as anti-metastatic lipids, which act in a synergistic and complementary fashion to enhance tumor cell growth, migration, survival and metastatic gene expression. Fourth, we provide exciting preclinical evidence that the combination treatment with LPCs and an AA signaling inhibitor BW-755C can significantly alleviate breast cancer bone metastasis. Fifth, our mouse genetic models reveal that bone metastasis can be markedly suppressed by β-catenin activation or PPARγ deficiency in the osteoclast lineage.

Lipid overabundance in the body has been shown to aggravate cancer metastasis (33). Intracellular lipid accumulation has been observed in many types of cancers including breast, brain, adrenal, and others (34,35). Highly proliferative cancer cells show a strong lipid avidity, which they satisfy by either increasing the uptake of exogenous lipids and lipoproteins or overactivating their endogenous biosynthesis (36), both of which can be heavily influenced by niche cell types in the tumor microenvironment. However, it has been largely overlooked how niche cells and tumor cells perform crosstalk using lipid-based language. What are the unique lipidomic profiles in the cancer-promoting niche cells and their secretome? What are the key lipid species from the niche cells that confer their pro-tumor and pro-metastatic functions? What are the functions of these lipids, metabolic or signaling? Using osteoclast niche cells and breast cancer cells, we begin to address these questions to understand how lipid osteoclastokines modulate cancer behavior and malignancy during bone metastasis.

AA and its metabolites generated by cyclooxygenases and lipoxygenases, such as prostaglandins and leukotrienes, have been reported to regulate a variety of biological processes including chemotaxis, cancer, and rheumatoid arthritis (37,38). In accordance with previous studies showing a pro-tumor role of AA signaling; here we report that AA also exerts a pro-metastatic role to enhance cancer cell migration, survival and metastatic gene expression. Moreover, we show that the levels of AA are elevated in the osteoclastic bone metastatic niche, as well as in circulation upon osteoclast activation of bone resorption, revealing AA as a novel player in the osteoclast-cancer cell vicious cycle. A potential explanation for why cancer cell proliferation was inhibited by BW-755C but unaltered by AA is that BW-755C may exert a broader effect to suppress the signaling of several unsaturated fatty acids including AA by inhibiting both cyclooxygenases and lipoxygenases.

LPCs have been implicated to exhibit anti-cancer effects. Reduced plasma LPC levels have been observed in patients with advanced cancer (39). In vitro studies indicated that LPCs can reduce cancer cell adhesion, viability and invasion (40,41). Their anti-cancer abilities are unique since LPCs do not target DNA but insert into plasma membrane to affect cellular processes such as adhesion and apoptosis through influencing several signaling pathways (40,41). Our study supports and extends these previous findings by showing: 1) the levels of LPCs are downregulated in the osteoclastic bone metastatic niche, as well as in circulation upon osteoclast
activation of bone resorption; 2) LPCs inhibit breast cancer cell growth, migration and survival in vitro; 3) treatment with LPCs alone, and more so in combination of an AA signaling inhibitor BW-755C, is sufficient to impede breast cancer bone metastasis in vivo. These new results further support LPCs as effective anti-cancer and anti-metastatic therapies.

Our data show that osteoclast activation or inhibition is sufficient to modulate circulating levels of AA and LPCs in vivo (Fig. 5A-F), supporting the physiological significance of our in vitro findings. Nonetheless, it is possible that these changes in AA and LPCs not only originate from osteoclasts but also contributed by other AA- and LPC-producing cells/tissues, such as brain, muscle and liver. We found that AA, LPCs and BW-755C do not directly alter osteoclast differentiation (Fig. S1), suggesting that these lipids mainly exert effects on tumor cells in the bone metastatic niche. However, they may indirectly alter bone resorption in vivo via the cancer cell-osteoclast vicious cycle such that their effects on cancer cells can relay to osteoclasts. Moreover, AA and LPCs may also modulate other cell types in the local and systemic cancer environment such as osteoblast and endothelial cells.

Previous studies show that factors released from bone matrix upon bone resorption play important roles in bone metastasis, our current work provide evidence for an additional and complementary mechanism in which osteoclast-derived lipids also polarize the bone metastatic niche to facilitate the cancer-osteoclast vicious cycle. Our examination of the specific roles of AA and LPCs in osteoclasts during bone metastasis in vivo is limited by the availability of AA-specific inhibitors and osteoclast-specific pharmacological targeting. In future studies, genetic strategies such as osteoclast-specific deletion or over-expression of AA- and LPC-specific metabolic enzymes may further elucidate the in vivo significance of this regulation.

It remains to be determined whether combo treatment with BW-755C and LPCs confers any therapeutic advantages compared with conventional osteoclast inhibitors bisphosphonates or denosumab. However, in light of the limitations of these current drugs such as lack of survival benefit as well as side effects including osteonecrosis of the jaw and renal toxicity, it is important to identify new signaling mechanisms and develop better therapeutic strategies. To this end, our studies using preclinical mouse models have demonstrated the functional significance of lipid osteoclastokines in bone metastasis and the therapeutic efficacy of their pharmacological targeting. Moreover, the levels of these osteoclast-regulated lipids in serum or biopsy, quantified by LC-MS or ELISA kits, may be used as prognostic indicators for cancer bone metastasis.

In conclusion, here we uncover, for the first time, a direct and lipid-mediated mechanism for how osteoclast enhances breast cancer cell metastatic features to promote the vicious cycle in bone metastasis. Provocatively, it is plausible that the unique profiles of lipid storage and lipid secretion in individual metastatic niche cell type may contribute to cancer metastasis organotropism. Our work suggests that the reprogramming of lipid composition by osteoclasts in the osseous milieu may cater to bone-seeking cancer cells and function as an attractant to facilitate cancer cell seeding and thriving in bone. Our findings reveal the exciting potential of lipids as novel prognostic and therapeutic targets for breast cancer bone metastasis.

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References


37. Villegas-Comonfort S, Castillo-Sanchez R, Serna-Marquez N, Cortes-Reynosa P, Salazar EP. Arachidonic acid promotes migration and invasion through a PI3K/Akt-dependent pathway in


**Figure 1. Osteoclast promotes breast cancer bone metastasis in vivo.** (A-G) Bone metastasis was blocked in osteoclastic β-catenin constitutive active (Oc-bCA) mice. (A, E) Kaplan Meier plot of time to metastasis over 8 weeks after cardiac injection of BoM-1833 cells. Similar results were obtained from Oc-bCA mice generated with PPARγ-tTA; TRE-cre (PT) (A) or Lysozyme-Cre (Ly) (E). Statistical analyses were performed with log-rank (Mantel-Cox) test. (B, F) Quantification of BLI signals. (C, G) Quantification of the number of bone metastatic sites. (D) Representative BLI images showing location and severity of bone metastases. (H) Kaplan Meier plot of time to metastasis showing that bone metastasis was diminished in osteoclastic β-catenin knockout (Oc-bKO) mice but exacerbated in osteoclastic β-catenin heterozygous (Oc-bHet) mice. Log-rank (Mantel-Cox) test showed p=0.0016 (**) for Oc-bKO vs. WT; p=0.0219 (*) for Oc-bHet vs. WT, and p<0.0001 for all 3 groups. (I) Quantification of BLI signals. (J) Quantification of the number of bone metastatic sites. (K-N) Bone metastasis was attenuated in osteoclastic PPARγ knockout (Oc-PPARγ-KO) mice. (K) Kaplan Meier plot of time to metastasis. Statistical analyses were performed with log-rank (Mantel-Cox) test. (L) Quantification of BLI signals. (M) Quantification of the number of bone metastatic sites. (N) BLI images. Error bars indicate SD; p values were from student t-test unless noted otherwise; *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001; n.s. non-significant.

**Figure 2. Lipid osteoclastokines enhance breast cancer bone metastasis.** (A) Cancer cell proliferation was enhanced by conditioned medium (CM) from WT bone marrow-differentiated osteoclasts (WT Oc CM) but not bCA differentiation cultures that failed to form osteoclast (bCA Oc CM), compared with CM from undifferentiated WT control cultures (WT Ctrl CM). BoM-1833 human breast cancer cell proliferation was quantified by luciferase readout (n=6). Comparisons with “WT Ctrl CM” at each time point were performed with student t-test, and p values are indicated in the graph. Two-way ANOVA analysis of all three groups showed p<0.0001 (**). (B) Cancer cell proliferation was enhanced by CM from RAW264.7 cell line-differentiated osteoclasts (Raw-Oc CM) compared with CM from undifferentiated RAW cells (Raw-Ctrl CM) (n=6). (C) Cancer cell migration was increased by WT Oc CM but not bCA Oc CM, compared with WT Ctrl CM. In a wound healing assay, cancer cell cultures were scratch-wounded to form a gap; cell migration was measured by gap closure 24 hrs later. Representative images are shown (n=4). (D) Cancer cell apoptosis was reduced by Oc CM vs. Ctrl CM,
quantified by Annexin V staining followed by flow cytometry (n=4). (E) Cancer cell seeding and proliferation on bone was increased by Oc CM vs. Ctrl CM, in a 3D bone metastasis model where cancer cells were co-cultured with a piece of calvarial bone in a roller tube for 7 days (n=6). (F) The ability of Oc CM to enhance cancer cell proliferation was intact after proteinase K (Pro.K) treatment and mainly stemmed from lipid factors (n=6). Comparison between Oc-CM and Ctrl-CM under each treatment condition was performed with student t-test, and p values are indicated in the graph. One-way ANOVA shows p value of non-significant. (G) The proliferation-enhancing effects of osteoclast-secreted lipids (Oc lipids) were specific for breast cancer cells but not for normal mammary epithelial cells, measured by MTT assay (n=6). (H) Oc lipids stimulated cancer cell migration equally well as Oc CM, quantified by trans-well assay (n=4). (I) Cancer cell expression of pro-metastatic genes was activated by Oc CM (n=3). ANOVA p<0.05. (J-K) Proliferation (J, n=6) and pro-metastatic gene expression (K, n=3) in Py8119 mouse mammary tumor cells were also enhanced by Oc lipids vs. Ctrl lipids. (L-N) Treatment with Oc lipids augmented breast cancer bone metastasis. Female C57BL/6J mice (8 week old, n=18) were intravenously injected with 50μg of Oc lipids or Ctrl lipids twice a week, started one week before Py8119 cancer cell intracardiac injection, and continued for two weeks afterwards. Bone metastases were analyzed two weeks after tumor cell inoculation. (L) Representative BLI images. (M) Quantification of the number of bone metastatic sites. (N) Quantification of bone metastasis BLI signals. Purified lipids were administrated at 2.5mg/kg in each mouse. Error bars indicate SD; p values were from student t-test unless noted otherwise; *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001; n.s. non-significant.

**Figure 3. Osteoclasts increase AA secretion to promote cancer cell metastatic features.** (A) Metabolomic profiling revealed increased AA and EPA secretion from osteoclasts. Untargeted LC-MS analysis was conducted under negative ionization mode to compare lipids isolated from the CM of osteoclasts vs. undifferentiated precursors (n=3). (B) Quantification of AA and EPA in Oc lipids and Ctrl lipids by targeted LC-MS analysis (n=3). (C) AA treatment increased cancer cell migration. In a trans-well assay, AA was added in the bottom chamber at the indicated concentration, and BoM-1833 cell migration from top chamber to bottom chamber was quantified by cell counting (n=4). (D) AA treatment decreased cancer cell apoptosis, quantified by Annexin V staining followed by flow cytometry (n=4). (E) AA treatment enhanced cancer cell expression of pro-metastatic genes (n=3). ANOVA p<0.05. (F) AA treatment did not alter cancer cell growth, for both MDA-MB231 (left) and BoM-1833 (right) cells, measured by luciferase output (n=6). Two-way ANOVA indicated p value of non-significant. (G) Py8119 cancer cell growth was unaltered by AA treatment but significantly reduced by the treatment of BW-755C (1.5μg/ml). Py8119 cell growth was measured by luciferase output (n=6). Comparisons with Ctrl at each time point were performed with student t-test, and p values are indicated in the graph. Two-way ANOVA shows p=0.0003 (**). (H) BW-755C significantly inhibited the growth of MDA-MB231 (left) and BoM-1833 (right) human breast cancer cells in a dose-dependent manner (n=6). Comparisons with 0μg/ml control were performed with student t-test, and p values are indicated in the graph. Two-way ANOVA shows p<0.0001 (****). (I-J) BW-755C treatment abolished the effects of Oc lipids (I) and AA (J) to increase cancer cell migration (n=4). BW-755C treatment was at 1.5μg/ml; AA treatment was at 10μM. Error bars indicate SD; p values were from student t-test unless noted otherwise; *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001; n.s. non-significant.
Figure 4. Osteoclasts decrease LPC secretion to promote cancer cell metastatic features. (A) Metabolomic profiling revealed decreased LPC 18:0 and LPC 16:0 secretions from osteoclasts. Untargeted LC-MS analysis was conducted under positive ionization mode to compare lipids isolated from the CM of osteoclasts vs. undifferentiated precursors (n=3). (B) Quantification of LPC 18:0 and LPC 16:0 in Oc lipids and Ctrl lipids by targeted LC-MS analysis (n=3). (C) LPC treatment decreased cancer cell migration. In a trans-well assay, LPC (10μM) was added in the bottom chamber, and BoM-1833 cell migration from top chamber to bottom chamber was quantified by cell counting (n=4). (D) LPC treatment (10μM) increased cancer cell apoptosis, quantified by Annexin V staining followed by flow cytometry (n=4). (E) LPC treatment increased Bax expression but decreased Bcl2 expression in a dose-dependent manner (n=3). (F-G) LPC treatment suppressed cancer cell growth, for both MDA-MB231 cells (F) and BoM-1833 cells (G), measured by luciferase output (n=6). (H) LPC treatment did not alter cancer cell expression of pro-metastatic genes (n=3). Error bars indicate SD; p values are from student t-test unless noted otherwise; *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001; n.s. non-significant; (E-G) ANOVA p<0.05.

Figure 5. Combination of BW-755C and LPCs suppresses bone metastasis in vivo. (A-C) Osteoclast activation increased AA but decreased LPCs in vivo. Mice were treated with RANKL (20μg/mouse) or PBS control once per week for 4 weeks, and then serum levels of CTX-1 (A), AA (B) and total LPC (C) were quantified by ELISA (n=5). (D-F) Osteoclast inhibition decreased AA but increased LPCs in vivo. Mice were treated with zoledronate (20μg/mouse) or PBS control once per week for 2 weeks, and then serum levels of CTX-1 (D), AA (E) and total LPC (F) were quantified by ELISA (n=5). (G-I) Female C57BL/6J mice (8-week-old, n=10) were treated with either BW-755C + LPC Combo (300μg BW-755C in 10% EtOH, and 5μg LPC 16:0 + 5ug LPC18:0 in 10% DMSO) or vehicle placebo (10% EtOH + 10% DMSO). The treatment was performed intravenously twice per week for one week before cardiac injection of Py8119 cancer cells, and then continued for three more weeks. Bone met quantifications at week 2 are shown as some mice died by week 3. (G) Representative BLI images from each experimental group. (H) Quantification of the number of bone metastatic sites. (I) Quantification of bone metastasis BLI signals. Error bars indicate SD; p values are from student t-test; *, p<0.05; ***, p<0.005. (J) A working model for how osteoclasts alter the secretion of lipids by increasing pro-metastatic AA and decreasing anti-metastatic LPCs to promote cancer cell metastasis to bone.
The figure (Fig. 1) illustrates the effects of different genotypes on bone metastasis and BLI (Bioluminescence Imaging) levels after cancer cell injection.

**A** Dorsal view showing the % bone metastasis after cancer cell injection for Oc-bCA (PT) (n=10) compared to WT (n=18).

**B** BLI levels (x10^6) and number of metastatic sites for Oc-bCA (PT) compared to WT.

**C** BLI levels (x10^6) and number of metastatic sites for Oc-bCA (Ly) compared to WT.

**D** Ventral view of WT and Oc-bCA (PT).

**E** Dorsal view showing the % bone metastasis after cancer cell injection for Oc-bCA (Ly) (n=8) compared to WT (n=9).

**F** BLI levels (x10^6) and number of metastatic sites for Oc-bCA (Ly) compared to WT.

**G** BLI levels (x10^6) and number of metastatic sites for Oc-bCA (PT) compared to WT.

**H** Dorsal view showing the % bone metastasis after cancer cell injection for Oc-bKO (n=9) and Oc-bHet (n=11) compared to WT (n=18).

**I** BLI levels (x10^6) and number of metastatic sites for Oc-bKO compared to WT.

**J** BLI levels (x10^6) and number of metastatic sites for Oc-bHEt compared to WT.

**K** Dorsal view showing the % bone metastasis after cancer cell injection for Oc-PPARg-KO (n=11) compared to WT (n=11).

**L** BLI levels (x10^6) and number of metastatic sites for Oc-PPARg-KO compared to WT.

**M** BLI levels (x10^6) and number of metastatic sites for Oc-bCA (PT) compared to WT.

**N** Ventral view of WT and Oc-PPARg-KO.
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**A**

Serum CTX-1 (ng/ml)

- Veh
- RANKL

**B**

Serum AA (ng/ml)

- Veh
- RANKL

**C**

Serum LPC (ng/ml)

- Veh
- RANKL

**D**

Serum CTX-1 (ng/ml)

- Veh
- Zolendronate

**E**

Serum AA (ng/ml)

- Veh
- Zolendronate

**F**

Serum LPC (ng/ml)

- Veh
- Zolendronate

**G**

BW-755C + LPCs Combo

**H**

# metastatic site

- Veh
- Combo

2 weeks after cardiac injection

**I**

BLU (x10^6)

- Veh
- Combo

2 weeks after cardiac injection

**J**

AA ↑
LPC ↓

↑ proliferation, migration, survival, metastatic genes

Osteoclast

Tumor cell

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