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Mechanical regulation of breast cancer migration and apoptosis via direct and indirect osteocyte signaling

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Abstract

Bone metastases, the migration of cancers to bone, occur in 65-80% of patients with advanced breast cancer. Metastasized cancer cells interact with cells such as the bone-resorbing osteoclasts to alter bone remodeling. Exercise, often suggested as an intervention for cancer patients, regulates bone remodeling via osteocytes. Osteocytes also signal to endothelial cells, which may affect cancer cell extravasation. Therefore, we hypothesize that mechanically stimulated osteocytes can regulate processes in breast cancer bone metastasis. To test this, we exposed osteocytes to oscillatory fluid flow in vitro using parallel-plate flow chambers. We observed that conditioned medium from flow-stimulated osteocytes increased migration (by 45%) and reduced apoptosis (by 12%) of breast cancer cells. Conditioned medium from osteoclasts conditioned in flowed osteocytes' conditioned medium reduced migration (by 47%) and increased apoptosis (by 55%) of cancer cells. Cancer cell trans-endothelial migration was reduced by 34% toward flowed osteocytes' conditioned medium. This difference was abolished with ICAM-1 or IL-6 neutralizing antibodies. Conditioned medium from endothelial cells conditioned in flowed osteocytes' conditioned medium increased cancer cell apoptosis by 29%. To summarize, this study demonstrated mechanically stimulated osteocytes' potential to affect breast cancer cells not only through direct signaling, but also through osteoclasts and endothelial cells. The anti-metastatic potential of the indirect signalings is particularly exciting since osteocytes are further away from metastasizing cancer cells than osteoclasts and endothelial cells. Future studies into the effect of bone mechanical loading on metastases and its mechanism will assist in designing cancer intervention programs that lowers the risk for bone metastases.

KEYWORDS
apoptosis, bone metastasis, breast cancer, extravasation, mechanical loading, migration, osteocyte

Shreyash Dalmia, Peter Gao, Jacob Young, and Kevin Middleton contributed equally to this work.

1 | INTRODUCTION

Bone metastasis, the spread of cancers from their primary tumor sites to bone, is a common and serious complication of cancers. It occurs in 65-80% of patients with advanced breast cancer,[1] and significantly increases the morbidity and mortality of patients. Metastasis happens when cancer cells break off from the primary tumor and travel in the blood or lymph vessels until they adhere or get physically arrested in narrow vessels.[2] Chemokines from the bone may then signal for cancer cells to extravasate and invade. The metastasized tumor cells are capable of interacting with and disturbing the balance between bone-forming osteoblasts and bone-resorbing osteoclasts.[3] This causes bone lesions and leads to symptoms such as bone fractures, pain, and hypercalcemia. It is often incurable once a secondary tumor is established in the bone, resulting in a 5-year survival rate of only 20%[4] that has not been significantly improved in the past 10 years.[5]

Since extravasation and secondary tumor growth requires complex interactions with other cells in the secondary site, there is a distinct preference in metastasis sites for different primary cancers.[2] As breast cancers most frequently metastasize to the bone, researchers have studied the interaction between breast cancer cells and multiple cells types in the bone. In particular, a vicious cycle has been identified between breast cancer cells and osteoclasts in which both tumor and lesions in the bone increase.[6] The cycle starts with breast cancer cells upregulating the recruitment and differentiation of osteoclasts by signaling directly to osteoclasts[7] and through osteoblasts.[8] The recruited osteoclasts can then support the cancer cells both by direct signaling[9] and by releasing growth factors from the bone matrix.[10] Therefore, many current treatments target osteoclasts. For example, denosumab blocks receptor activator of nuclear factor-kB ligand (RANKL), an essential factor for osteoclastogenesis,[11] and has been demonstrated to successfully reduce bone turnover in patients with breast cancer bone metastasis.[12]

However, osteocytes, an important regulator of bone remodeling,[13] have only recently been studied for their potential role in regulating bone metastasis.[14–16] Osteocytes are the most abundant cells in the bone.[17] They reside in lacunae in the bone that are interconnected by canaliculi. When the bone is mechanically loaded, such as during exercise, oscillatory fluid flow (OFF) is generated in this lacunar-canalicular system.[18] Osteocytes are known to signal to other cells in the bone when stimulated with a physiological levels of fluid shear stress (0.5-3 Pa).[13,19] Specifically, bone resorption by osteoclasts is downregulated,[20] while bone formation by osteoblasts is upregulated[21] This demonstrates the capability of osteocytes to regulate the bone-remodeling balance and break the vicious cycle of bone metastasis. Furthermore, mechanically stimulated osteocytes can signal to endothelial cells and affect angiogenesis[22] as well as their expression of intercellular adhesion molecule 1 (ICAM-1) by lowering interleukin 6 (IL-6) secretion.[23] This, consequently, have a potential regulatory impact on cancer cell extravasation. In fact, since endothelial cells will be in greater abundance and at a closer distance to the metastasizing breast cancer cells in blood vessels than osteoclasts and osteocytes,[24] this signaling pathway may have more impact before the vicious cycle is established.

Therefore, we hypothesize that mechanical loading may regulate processes in bone metastases via osteocyte signaling. Mechanically stimulated osteocytes could affect cancer cell extravasation as well as signaling to osteoclasts to alter their interaction with metastasizing cells. Although numerous studies have been performed to investigate the role of osteoclasts[10] and endothelial cells[25] in bone metastasis, studies on osteocytes, the major cells in the bone that regulate both osteoclasts[21] and endothelial cells,[23,24] remains limited.[14–16] This project aims to investigate the effect of osteocytes' response to OFF on breast cancer migration and apoptosis through 1) direct signaling to the cancer cells, and indirect signaling mediated by 2) osteoclasts and 3) endothelial cells. Since endothelial cells comprise the important first barrier encountered by metastasizing cancer cells in blood vessels, we investigated the mechanisms responsible for the observed difference in breast cancer cell trans-endothelial migration toward conditioned media (CM) from mechanically stimulated osteocytes. This could eventually lead to an optimized exercise regimen that not only improves patients' quality of life,[26] but also lowers their risk for bone metastasis.

2 | MATERIAL AND METHODS

2.1 | Cell cultures

Highly metastatic MDA-MB-231 human breast cancer cells (catalog number HTB-26; ATCC, Manassas, VA) were cultured in Kaighn's modification of Ham's F-12 media (21127022; Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (12483-020; Gibco) and 1% penicillin-streptomycin (P/S) (15140122; Gibco). Non-metastatic MCF-7 human breast cancer cells (HTB-22; ATCC) were used as a control. They were cultured in Minimum Essential Medium Eagle (M4655; Sigma-Aldrich, St. Louis, MO), supplemented with 10% FBS (12483-020; Gibco) and 1% P/S. MLO-Y4 osteocyte-like cells (a gift from Dr. Bonewald, Indiana University, Indianapolis, IN) were cultured in α-Modified Essential Medium (12571071; Gibco), supplemented with 2.5% FBS (12483-020; Gibco), 2.5% calf serum (16010-159; Gibco), and 1% P/S on petri dishes or glass slides coated with 0.15 mg/mL type-I rat tail collagen (354236; Coring Life Science, Lowell, CA). RAW264.7 osteoclast precursors (TIB-71; ATCC) were cultured in Dulbecco's

Modified Eagle Medium (D5671; Sigma-Aldrich) supplemented with 10% FBS (SH30071.03; HyClone, Logan, UT), 2% L-Glutamine (25030081; Gibco), and 1% P/S. Human umbilical vein endothelial cells (HUVECs) (a gift from Dr Young and Dr Simmons, University of Toronto, Toronto, ON, Canada) were cultured in endothelial cell growth media (CC-3162; Lonza, Basel, Switzerland). All cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

2.2 | Oscillatory fluid flow on osteocytes

MLO-Y4 osteocyte-like cells were cultured on collagen-coated glass slides until 80% confluency was achieved. The glass slides were placed in parallel-plate flow chambers[27] that were filled with MLO-Y4 growth media and connected to syringes on a linear actuator to generate 1 Hz OFF with peak shear stress of 1 Pa. After 2 h of flow, the glass slides were removed from the flow chambers and placed in 10 mL MLO-Y4 growth medium to be incubated for 24 h. Conditioned medium (CM), containing soluble cytokines secreted by MLO-Y4 cells, was then extracted (MLO-Y4 CM).

A peak fluid shear stress of 1 Pa was selected because osteocytes residing in lacunae are estimated to experience shear stress of 0.5-3 Pa physiologically,[19] and 1 Pa approximately simulates mechanical loading during a mild exercise. The timings of 2-h flow and 24-h post-flow incubation were chosen based on studies demonstrating that MLO-Y4 CM collected under these conditions can regulate osteoclastogenesis.[20]

2.3 | Transwell migration assay

Breast cancer cells (MDA-MB-231 or MCF-7 cells) were stained with cell tracker green (C2925; Life Technologies, Carlsbad, CA) on the day of experiment. 1200 μL of CM was added per well in a 12-well plate. Transwells with an 8 μm pore-size semi-permeable membrane (665638; Greiner Bio-One, Cassina de Pecchi, Italy) were then placed in the wells. 200 μL of 400 000 cells/mL cell suspension was added to Transwells and allowed to migrate for 6 h. Cells were then fixed with 10% neutral buffered formalin (HT501128; Sigma-Aldrich), and the top of the Transwells was scrapped with cotton swabs to remove cells that did not migrate. Five images were taken per Transwell. The number of migrated cells was quantified with ImageJ (NIH, Bethesda, MD).

2.4 | Osteoclastogenesis

RAW264.7 osteoclast precursors were cultured in 50% growth media and 50% MLO-Y4 CM in 12-well plates. 10 ng/mL RANKL (462-TR-010; R&D Systems, Minneapolis, MN) was added because MLO-Y4 CM can regulate osteoclastogenesis,[21] but is not enough to stimulate osteoclastogenesis alone.[28] Medium was changed daily after day 3. At day 6, medium was replaced with 3 mL of RAW264.7 growth media to ensure the collected CM did not contain factors secreted by MLO-Y4 cells. At day 7, medium was collected (RAW264.7 CM) and cells were fixed and stained for tartrate-resistant acid phosphatases (TRAP) using a leukocyte acid phosphatase kit (387A; Sigma-Aldrich). Osteoclasts were identified as TRAP-positive cells containing three or more nuclei.[29] The total number of osteoclasts in a well was quantified.

2.5 | Trans-endothelial migration

Transwells were coated with 0.15 mg/mL type-I rat tail collagen then seeded with HUVECs. When HUVECs were 100% confluent, 1200 μL of MLO-Y4 CM was added to the wells and 200 μL of cell tracker green-stained breast cancer cell suspension (400 000 cells/mL) was added to the Transwells. After 18 h of migration, cells were fixed 10% neutral buffered formalin, and the cells that have not migrated were scrapped off with a cotton swab. Five images were taken per well and number of migrated cells was quantified using imagej. An extra Transwell seeded with HUVECs was stained with anti-VE-Cadherin antibody (ab33168; abcam, Cambridge, UK) for each set of experiment to confirm that an intact layer was formed before performing the trans-endothelial migration.

Blocking experiments were performed similarly to previously described.[23] Briefly, 1 μg/mL mouse anti-human ICAM-1 antibody (BBA3; R&D Systems) was added to endothelial cells in MLO-Y4 CM for 1 h prior to the addition of breast cancer cells, and 2 μg/mL rat anti-mouse IL-6 antibody (MAB406; R&D Systems) was added to MLO-Y4 CM 1 h prior to addition to HUVECs.

2.6 | Apoptosis

MDA-MB-231 cells were cultured in 50% regular growth media and 50% CM in 96-well plates for 48 h. Apoptosis of these cells was then quantified by staining with 5% APOPercentage dye (A1000; Biocolor, Carrickfergus, UK) for 1 h. For assaying apoptosis of MDA-MB-231 cells in CM from HUVECs conditioned in MLO-Y4 CM, HUVECs were first cultured in 96-well plates until 100% confluency was achieved. 100 μL of MLO-Y4 CM and 100 μL of HUVEC growth medium was then added to the HUVECs. After 24 h, medium was removed and HUVECs were cultured in 200 μL of HUVEC growth medium for 12 h. Finally, 100 μL of this medium (HUVEC CM) was collected and added to MDA-MB-231 cells with 100 μL of MDA-MB-231 growth medium. Apoptosis of these MDA-MB-231 cells was quantified in 48 h.

2.7 | Statistics

Results were normalized to controls (results from MDA-MB-231 cells in CM when the MLO-Y4 cells were seeded on glass slides but not placed in flow chambers) to account for potential differences in experimental conditions. At least three individual experiments were run with at least four samples for each group in one experiment. Average from an individual experiment is treated as 1 data-point in statistical analysis because this approximates the data as normally distributed (central limit theorem). Student's *t*-test (2-tail, paired) was used to test significance between flow and no-flow groups (significance was taken at $\alpha = 0.05$).

3 | RESULTS

3.1 | Direct effect of flow-stimulated osteocytes on breast cancer cells

The migration of highly metastatic MDA-MB-231 breast cancer cells toward MLO-Y4 CM was 45% higher when the MLO-Y4 osteocyte-like cells were stimulated with OFF (1 Pa peak shear stress, 1 Hz, 2 h) (Figure 1A). Meanwhile, the less-migratory MCF-7 breast cancer cells did not migrate significantly toward MLO-Y4 CM, either when the MLO-Y4 cells were stimulated to flow or not (Figure 1A). Therefore, as MCF-7 cells would not reach osteocytes due to the lack of migration, apoptosis in MLO-Y4 CM was examined only for MDA-MB-231 cells and not MCF-7 cells.

MDA-MB-231 cells were then cultured in 50% MLO-Y4 CM and 50% MDA-MB-231 growth media for 48 h and tested for their apoptosis. With the APOPercentage stain, it was shown that the apoptosis of MDA-MB-231 cells dropped by 12% when they were cultured in the CM from flow-stimulated MLO-Y4 cells (Figure 1C).

3.2 | Effect of flow-stimulated osteocytes on breast cancer cells via osteoclasts

Since studies have shown that osteocytes signal to osteoclasts[20] and that osteoclasts affect cancer cell activities,[9] it is

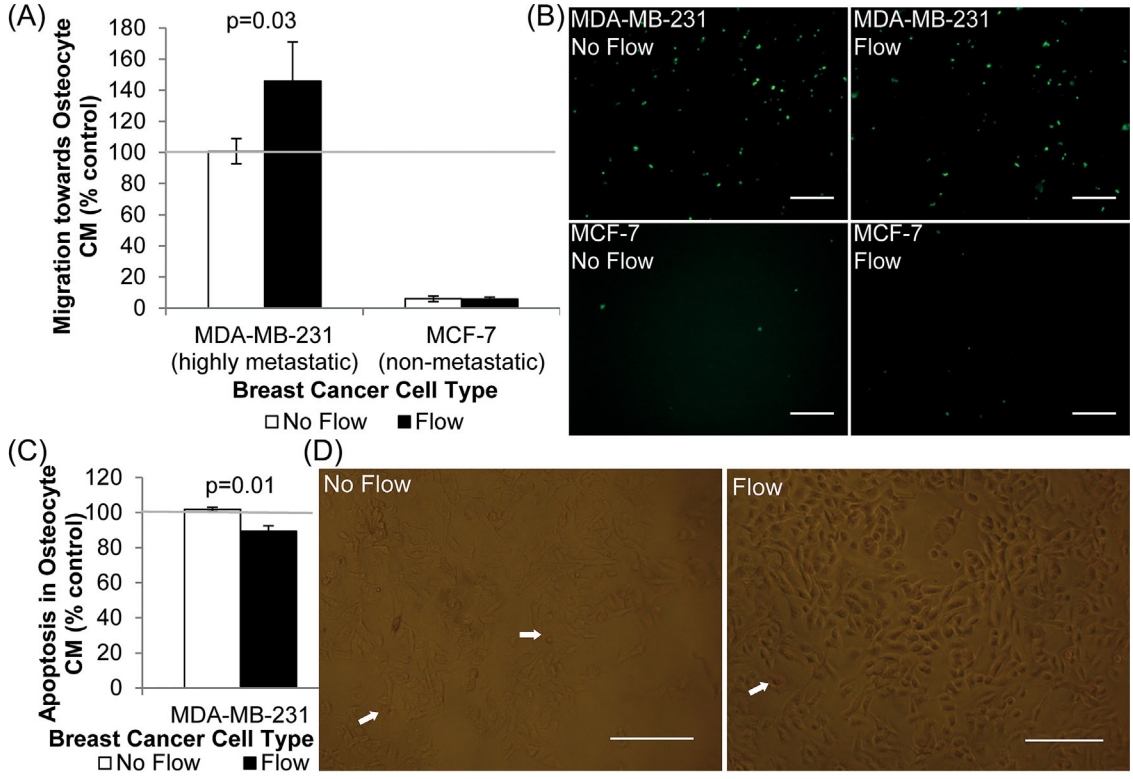

FIGURE 1 Breast cancer cell migration toward and apoptosis in osteocyte CM. A, Number of MDA-MB-231 and MCF-7 cells that migrated across Transwell toward MLO-Y4 CM, normalized to controls (MDA-MB-231 migration toward CM from MLO-Y4 cells not placed in flow chambers). Data are presented as mean ± standard deviation, $n = 3$ experiments (14 samples) for MDA-MB-231, $n = 2$ experiments (12 samples) for MCF-7. B, Sample paired images of cell tracker green-stained MDA-MB-231 and MCF-7 cell migration toward no-flow and flow MLO-Y4 CM. Scale bar = 200 μm. C, Percentage of apoptotic MDA-MB-231 cells in MLO-Y4 CM, normalized to controls (MDA-MB-231 cell apoptosis in CM from MLO-Y4 cells not placed in flow chambers). Data are presented as mean ± standard deviation, $n = 3$ experiments (14 samples). D, Sample paired images of MDA-MB-231 cells stained with APOPercentage after culturing in no-flow and flow MLO-Y4 CM. Arrows indicate sample apoptotic cells in pink. Scale bar = 200 μm

important to investigate whether osteoclasts mediate the signaling of flow-stimulated osteocytes to breast cancer cells. First, using TRAP-stain, we confirmed that 44% fewer osteoclasts differentiated when RAW264.7 cells were cultured in CM from MLO-Y4 cells stimulated with flow (Figure 2).

Subsequently, breast cancer cell migration toward CM from RAW264.7 cells cultured in MLO-Y4 CM was assayed. Migration of highly metastatic MDA-MB-231 cells toward RAW264.7 CM was 47% less when the MLO-Y4 cells were stimulated with flow, while the non-metastatic MCF-7 cells did not migrate toward RAW264.7 CM (Figure 3A). Therefore, the apoptosis of MCF-7 cells in RAW264.7 CM was not quantified as they will not migrate toward osteoclasts. Apoptosis rate of MDA-MB-231 cells in RAW264.7 CM was shown to be 55% higher when MLO-Y4 cells were stimulated with flow (Figure 3C).

3.3 | Effect of flow-stimulated osteocytes on breast cancer cells via endothelial cells

Although osteoclasts are potent mediators of flow-stimulated osteocytes' signaling to breast cancer cells, there are a relatively small number of osteoclasts at the bone surface[30] before the bone metastasis vicious cycle is established. On the other hand, endothelial cells are present in greater number and in close proximity to the metastasizing cancer cells in blood vessels.[24] They play a major role in metastasis[25] and are regulated by osteocytes, as shown by our previous studies.[22,23] Therefore, we here examined whether endothelial cells mediate osteocytes' effect on metastasis.

The trans-endothelial migration of highly metastatic MDA-MB-231 cells toward MLO-Y4 CM was reduced by 34% when MLO-Y4 cells were stimulated with flow (Figure 4A). An extra Transwell seeded with HUVEC was stained for VE-cadherin to confirm that an intact endothelial layer was achieved for each set of experiment (Figure 4B). Since the non-metastatic MCF-7 cells did not migrate toward MLO-Y4 CM without the presence of endothelial cells, their trans-endothelial migration was not examined. Apoptosis rate of MDA-MB-231 cells in HUVEC CM was shown to be 29% less when MLO-Y4 cells were stimulated with flow (Figure 5).

As endothelium is the first barrier encountered by breast cancer cells circulating in blood vessels during metastasis, we also investigated the factors responsible for the effect we observed on trans-endothelial migration. Because mechanically stimulated MLO-Y4 cells are less apoptotic[22] and secrete less IL-6,[23] MLO-Y4 CM from these cells reduces the expression of ICAM-1 by endothelial cells,[23] and subsequent MDA-MB-231 cancer cell extravasation.[31] Here, we administered anti-IL-6 and anti-ICAM-1 neutralizing antibodies to investigate this pathway. Application of anti-ICAM-1 and anti-IL-6 (Figure 4) antibodies not only significantly reduced the overall trans-endothelial migration of MDA-MB-231 cells, but also abolished the increased trans-endothelial migration toward MLO-Y4 CM from static MLO-Y4 cells.

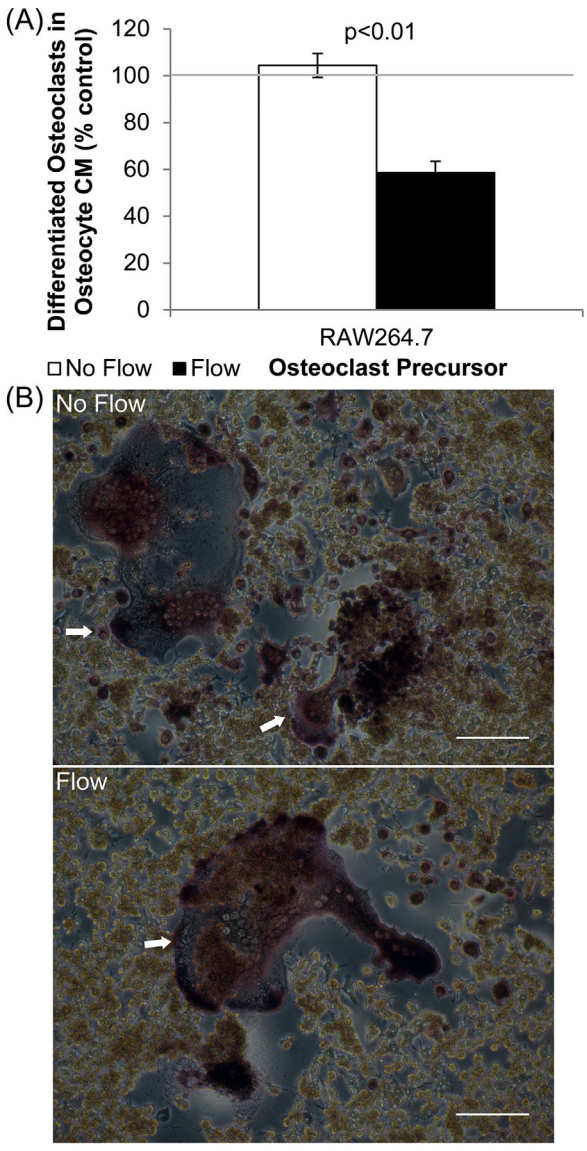

FIGURE 2 Osteoclastogenesis in osteocyte CM. A, Number of differentiated osteoclasts, normalized to controls (RAW264.7 osteoclastogenesis in CM from MLO-Y4 cells not placed in flow chambers). Data are presented as mean ± standard deviation, $n = 3$ experiments (19 samples). B, Sample paired images of differentiated osteoclasts after culturing in no-flow and flow MLO-Y4 CM. Osteoclasts are identified as TRAP-positive (purple-stained) multinucleated cells, indicated by arrows. Scale bar = 100 μm

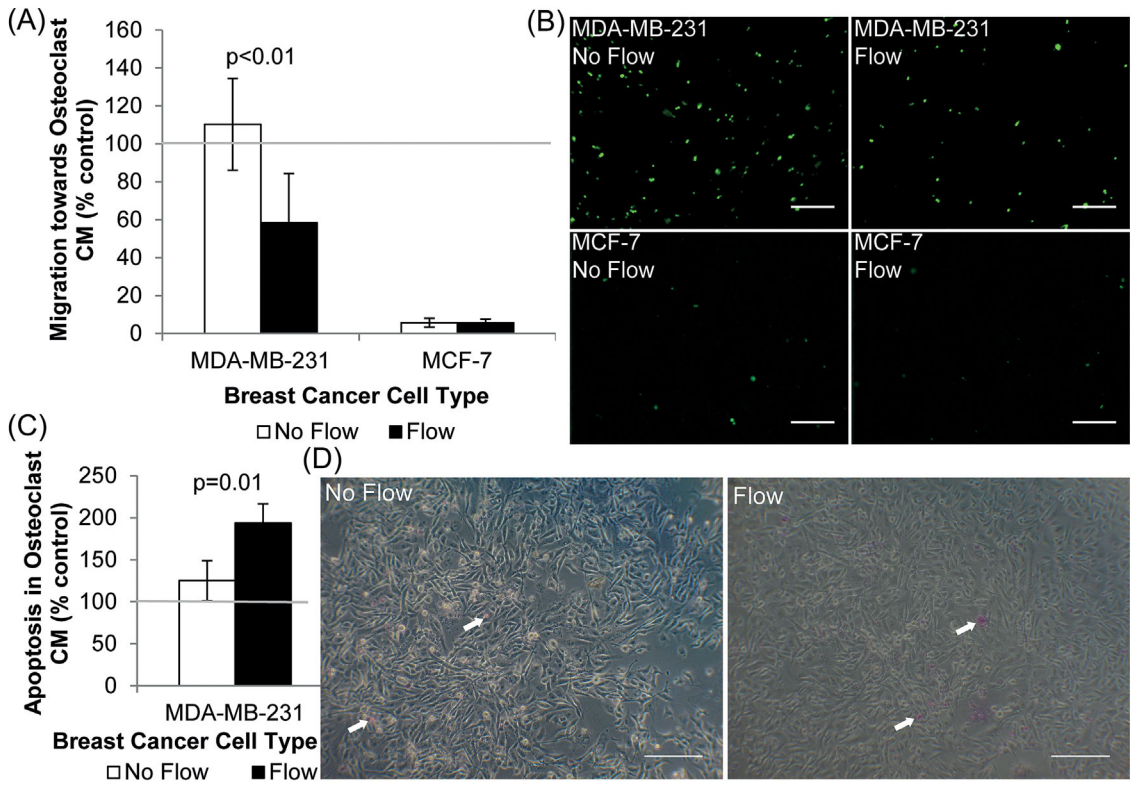

FIGURE 3 Breast cancer cell migration toward and apoptosis in CM from osteoclasts cultured in osteocyte CM. A, Number of MDA-MB-231 and MCF-7 cells that migrated through Transwell toward RAW264.7 CM, normalized to controls (MDA-MB-231 migration toward CM from RAW264.7 cells cultured in CM from MLO-Y4 cells not placed in flow chambers). Data are presented as mean ± standard deviation, $n = 3$ experiments (20 samples) for MDA-MB-231, $n = 2$ experiments (12 samples) for MCF-7. B, Sample paired images of cell tracker green-stained MDA-MB-231 and MCF-7 cell migration toward RAW264.7 CM. Scale bar = 200 μm. C, Number of apoptotic MDA-MB-231 cells in CM from RAW264.7 cells cultured in MLO-Y4 CM, normalized to controls (MDA-MB-231 apoptosis in CM from RAW264.7 cells cultured in CM from MLO-Y4 cells not placed in flow chambers). Data are presented as mean ± standard deviation, $n = 3$ experiments (14 samples). D, Sample paired images of MDA-MB-231 cells stained with APO Percentage after culturing RAW264.7 CM when MLO-Y4 cells were static and stimulated with flow. Arrows indicate sample apoptotic cells in pink. Scale bar = 200 μm

4 | DISCUSSION

In this study, we investigated whether mechanical loading on osteocytes can affect cancer cell migration and apoptosis. We observed that, indeed, signals from mechanically stimulated osteocytes can have potent regulatory effect on cancer cells. Specifically, as demonstrated in the results and summarized in Figure 6, direct and cell-mediated signaling from osteocytes exposed to OFF (1 Pa peak shear stress, 1 Hz, 2 h) seem to have opposite effects on breast cancer cell migration and apoptosis. Applying CM from flow-stimulated MLO-Y4 osteocyte-like cells directly to metastatic MDA-MB-231 breast cancer cells increased their migration (Figure 1A) while reducing their apoptosis (Figure 1C). However, CM from RAW264.7 osteoclasts cultured in CM from flow-stimulated osteocytes (Figure 2) reduced breast cancer cells' migration (Figure 3A) while increasing their apoptosis (Figure 3C). As well, CM from flow-stimulated osteocytes reduced the trans-endothelial migration of breast cancer cells due to the lower expression of IL-6 by osteocytes and subsequent ICAM-1 by HUVECs (Figure 4). CM from HUVECs cultured in CM from flow-stimulated osteocytes also increased breast cancer cell apoptosis (Figure 5). These suggest an anti-metastatic potential of flow-stimulated osteocytes mediated by osteoclasts and endothelial cells.

There are many factors secreted by flow-stimulated osteocytes that may have a direct impact on cancer cell migration and apoptosis. For example, flow-stimulated osteocytes' decreased RANKL[20] and increased adenosine triphosphate (ATP)[21] secretion could inhibit cancer cell migration,[16,32] whereas their increased vascular endothelial growth factor (VEGF)[22] and osteopontin[33] secretion could increase cancer cell migration.[34,35] We here demonstrated that CM from osteocytes exposed to OFF increased breast cancer cells' migration (Figure 1A) while reducing their apoptosis (Figure 1C). Nevertheless, since we believe osteocytes embedded in the bone matrix may not be at an ideal location to signal directly to the metastasizing cancer

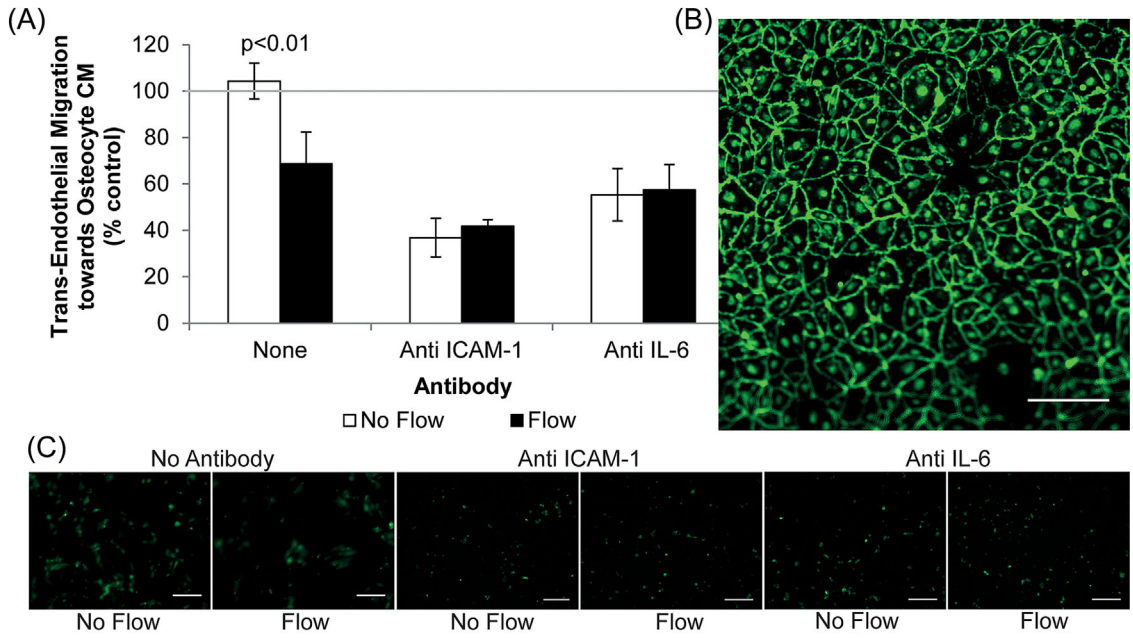

FIGURE 4 Breast cancer cell trans-endothelial migration toward osteocyte CM, with and without ICAM-1 and IL-6 neutralizing antibodies. A, Number of MDA-MB-231 cells migrated across a layer of HUVECs on a Transwell toward MLO-Y4 CM, normalized to controls (MDA-MB-231 cell trans-endothelial migration toward CM from MLO-Y4 cells not placed in flow chambers). Data are presented as mean ± standard deviation, $n = 4$ experiments (23 samples) for no-antibody, $n = 4$ experiments (18 samples) for ICAM-1 antibody, $n = 3$ experiments (12 samples) for IL-6 antibody. B, Sample image of an intact layer of HUVECs on Transwells stained for VE-cadherin. Scale bar = 200 μm. C, Sample paired images of cell tracker green-stained MDA-MB-231 cell trans-endothelial migration toward no-flow and flow MLO-Y4 CM, with and without ICAM-1 and IL-6 neutralizing antibodies. Scale bar = 200 μm

cells in vessels, we did not investigate further into this mechanism. Our observation seems to contradict the Zhou et al study, which demonstrated that osteocytes' increased connexin hemichannels expression and subsequent ATP release when stimulated with uni-directional flow can inhibit breast cancer cell migration.[15] However, there are significant differences on the experimental design between this study and our study. First of all, we apply OFF, which more closely simulates bone fluid flow[19] and has been shown to affect osteocyte signalling in a different way from other flow profiles,[27] instead of uni-directional flow. Secondly, we incubated osteocytes for 24 h post-flow before CM collection. As ATP molecules are degraded fast once outside of cells,[36] the ATPs secreted in response to flow may have been broken down during the 24-h incubation.

As previously mentioned, although osteocytes are the major population of cells in the bone, they reside within the bone matrix, and thus other cells that are located closer to metastasizing cancer cells in the blood vessels may mediate the signaling from osteocytes. In particular, osteoclasts, identified as a major player in the vicious cycle of bone metastasis, interact closely with cancer cells[9] and osteocytes.[20] They are located in the bone remodeling units that are close to the sinusoid blood vessels,[37] which has a slow blood flow and, therefore, is a common site for breast cancer cells to adhere and extravasate.[38] Consequently, their proximity and known interaction with cancer cells and osteocytes make them a potential candidate for mediating of osteocytes' effect on cancer cells.

In this study, we confirmed that flow-stimulated osteocytes reduce osteoclastogenesis (Figure 2), and demonstrated that CM from these osteoclasts reduced migration (Figure 3A) and increased apoptosis (Figure 3C) of breast cancer cells. This suggested anti-tumor potential of bone mechanical loading aligns well with a previous in vivo study that showed a reduction in tumor size when mice with breast cancer cells injected into their tibia are subjected to tibial loading.[39] Since we have verified that flow-stimulated osteocytes reduce osteoclastogenesis, we hypothesize that this observed osteoclast-mediated effect of osteocytes on breast cancer cells is a result of osteoclasts secreting various chemoattractants and growth factors. This is supported by the Krzeszinski et al study, which recently identified arachidonic acid as the osteoclast-secreted lipid cytokine responsible for attracting breast cancer cells.[9] The osteoclast-secreted factors that recruit osteoblasts for bone remodeling balance[37] could potentially contribute as well, since breast cancer cells are known to express signaling factors and receptors usually unique to bone cells.[40] For example, sphingosine-1-phosphate (S1P) is secreted by osteoclasts[41] and is known to support cancer cell migration and growth.[42]

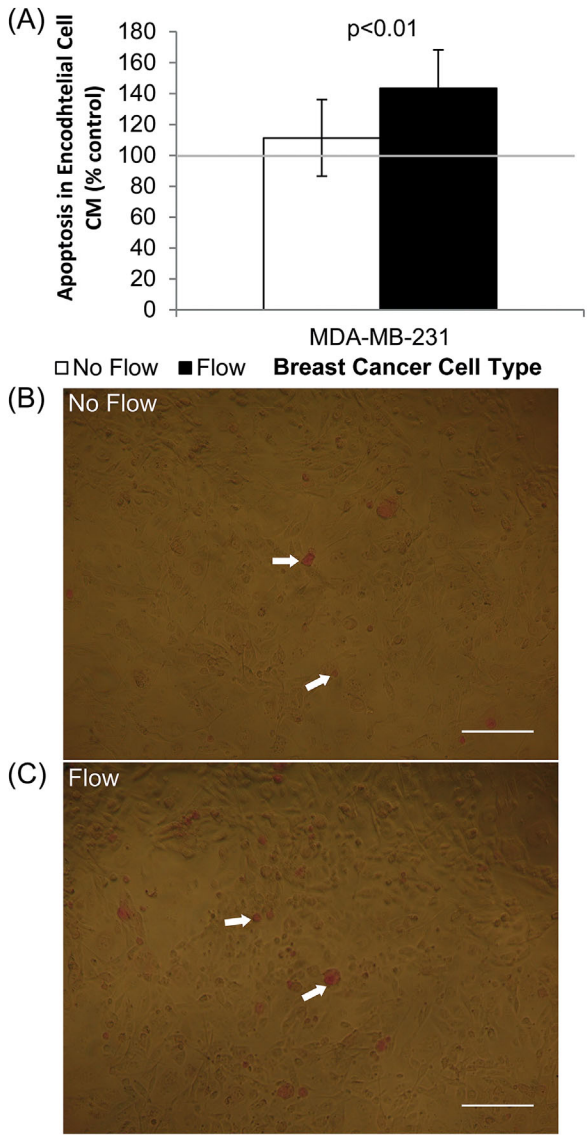

FIGURE 5 Breast cancer cell apoptosis in CM from endothelial cells cultured in osteocyte CM. A, Apoptosis of MDA-MB-231 cells in CM from HUVECs cultured in MLO-Y4 CM, normalized to controls (MDA-MB-231 apoptosis in CM from HUVECs cultured in CM from MLO-Y4 cells not placed in flow chambers). Data are presented as mean ± standard deviation, $n = 3$ experiments (16 samples). B, Sample paired images of MDA-MB-231 cells stained with APO Percentage after culturing in HUVEC CM when MLO-Y4 cells were static and stimulated with flow. Arrows indicate sample apoptotic cells in pink. Scale bar = 200 μm

However, more investigation would be needed to identify the osteoclast-secreted factors responsible for our observation.

On the other hand, osteoblasts have also been shown to regulate metastasizing breast cancer cells directly and indirectly through osteoclasts.[43] A study demonstrated osteoblasts' potential to modulate the high bone turnover rate caused by metastatic breast cancer cells.[44] In vivo, mechanically stimulated osteocytes reduce sclerostin secretion,[21] which in turn increase osteoblastic bone formation through BMP and Wnt signaling pathways,[45,46] as well as reduce osteoclastic bone resorption through RANKL-dependent pathway.[47] This may help to restore the altered bone turnover rate in bone metastasis. Interestingly, breast cancer cells can also express sclerostin to reduce osteoblastic bone formation.[48] Although the role of sclerostin and osteoblasts in bone metastasis is highly interesting, very little is known regarding how osteocyte-derived sclerostin affects bone metastasis. Unfortunately, our model could not investigate this due to the low sclerostin expression by MLO-Y4 cells. New osteocyte cell-lines that have high expression of sclerostin, such as the OCY454 cells,[49] may be used for further investigation.

Moreover, it should be noted that the non-metastatic breast cancer cell-line MCF-7 did not migrate toward CM from osteocytes (Figure 1A) or osteoclasts (Figure 3A) under the same experimental condition as the highly metastatic MDA-MB-231 cells. This confirms a previous study that demonstrated weakly invasive and migratory property of MCF-7 cells in vitro and in vivo.[50] More importantly, this confirms that CM from flow-stimulated osteocytes and from conditioned osteoclasts specifically attracts MDA-MB-231 cells, which have been shown to metastasize to the bone in vivo.[51] Since MCF-7 cells did not migrate toward CM, their extravasation and apoptosis were not studied.

Although osteoclasts on the bone remodeling surface are in the vicinity of, and interact with, both osteocytes ad metastasizing cancer cells, there are relatively few osteoclasts in normal bone before the bone metastasis vicious cycle is established.[30] Therefore, we shifted our focus to endothelial cells. Endothelial cells are present in the greatest number and closest proximity to breast cancer cells in bone marrow sinusoids,[24] a common site for cancer cells to adhere and extravasate due to reduced blood flow.[38] Furthermore, extravasation across endothelial cells into the bone is an essential step in bone metastasis; and flow-stimulated osteocytes are capable of regulating endothelial cells.[22,23] Our study showed that, on top of reducing osteoclasts' support for cancer cells, CM from flow-stimulated osteocytes also reduced the trans-endothelial migration of breast cancer cells (Figure 4A). CM from endothelial cells conditioned in CM from flow-stimulated osteocytes increased breast cancer cell apoptosis as well (Figure 5). These further strengthen the anti-metastatic potential of bone mechanical loading. There are several factors in CM from flow-stimulated osteocytes that may affect endothelial permeability, which would in turn affect cancer cell extravasation. For example, the increase in prostaglandin E2 (PGE-2) production by mechanically loaded osteocytes[52] may result in reduced endothelial permeability.[53] The reduced apoptosis of flow-stimulated osteocytes[22] also leads to lower IL-6 secretion by osteocytes and lower ICAM-1 expression on endothelial cells.[23] This will then reduce the adhesion and trans-endothelial migration of cancer cells.[31] Here, we showed that the application of anti-IL-6 and anti-ICAM-1 antibodies significantly reduced the trans-endothelial migration of MDA-MB-231 cells and abolished the

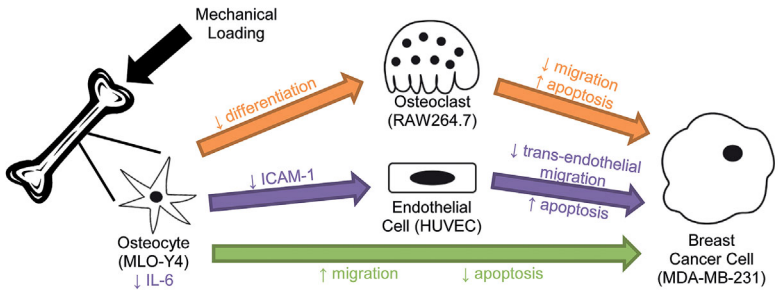

FIGURE 6 Direct and indirect signaling from flow-stimulated osteocytes on breast cancer cell migration and apoptosis. Applying CM from flow-stimulated osteocytes directly to breast cancer cells seem to be pro-metastatic, by increasing breast cancer cell migration and reducing apoptosis (Figure 1). However, incorporating osteoclasts and endothelial cells suggests an anti-metastatic potential of flow-stimulated osteocytes, by reducing breast cancer cell migration (Figure 3A) and extravasation (Figure 4), while increasing their apoptosis (Figures 3C and 5). The difference in breast cancer cell trans-endothelial migration was abolished by IL-6 or ICAM-1 neutralizing antibodies (Figure 4)

difference in MLO-Y4 CM from static and flow-stimulated MLO-Y4 cells (Figure 4A). This demonstrates the important regulatory role of mechanically stimulated osteocytes on breast cancer extravasation through osteocyte-secreted IL-6 and endothelial-expressed ICAM-1.

There are a few limitations in this study due to the use of cell-lines. MLO-Y4 osteocytes and RAW264.7 osteoclast precursors used in this study are of mice origins, while MDA-MB-231 and MCF-7 breast cancer cells are of human origins. However, both human breast cancer cell types are very well-established and have been studied extensively in mice models. Osteoclasts differentiated from RAW264.7 cells have also been used in numerous studies with MDA-MB-231 cells[9,54] or MLO-Y4 cells.[19,55] In addition, as mentioned above, the low sclerostin expression by MLO-Y4 cells prevented us from studying the highly interesting role of sclerostin and osteoblasts in bone metastasis. However, although MLO-Y4 cells are not ideal, it is the most well-understood osteocyte cell-line.[56] The use of established cell-lines as opposed to primary cells allows for a better control of difference between experiments. Furthermore, primary osteocytes are difficult to obtain and proliferate in vitro as they are terminally differentiated. Therefore, although these established cell-lines come with limitations, using them granted us the capability to better control experimental variabilities and to focus on the factors of interest. The approach of sequential conditioning instead of a direct co-culture involving all cell populations or an in vivo system, although may be less physiological, also allowed us to isolate effects and investigate only the interaction between two cell types at a time. Similarly, although it would be interesting to observe the combined effect of osteoclasts and endothelial cells on breast cancer cells, it would be difficult to isolate factors secreted from distinct cell populations and to investigate mechanisms. In addition, as the number of osteoclasts is low before bone metastasis is established, the effect of osteoclasts may be over-represented in a system of combined CM or co-culture. Moreover, the use of CM means only soluble factors could have an impact. This is more realistic since it is unlikely for the cancer cells traveling in the blood vessels to have direct contacts with osteocytes in the bone matrices or differentiated osteoclasts on the bone surfaces.

In summary, this research explored the effect of mechanically loaded osteocytes, both directly and indirectly through other cells in the bone microenvironment, on breast cancer migration and apoptosis in vitro. Research into the effect of osteocytes on breast cancer cells gives us insight into the impact of exercise on bone metastasis. Investigating the mechanisms of this phenomenon will provide potential drug targets that can be used in combination with exercise routines to improve patients' quality of life while reducing their risk for bone metastasis.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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