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RESEARCH ARTICLE

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Mechanically stimulated osteocytes reduce the bone-metastatic potential of breast cancer cells in vitro by signaling through endothelial cells

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Abstract

Bone metastases occur in 65% to 75% of patients with advanced breast cancer and significantly worsen their survival and quality of life. We previously showed that conditioned medium (CM) from osteocytes stimulated with oscillatory fluid flow, mimicking bone mechanical loading during routine physical activities, reduced the transendothelial migration of breast cancer cells. Endothelial cells are situated at an ideal location to mediate signals between osteocytes in the bone matrix and metastasizing cancer cells in the blood vessels. In this study, we investigated the specific effects of flow-stimulated osteocytes on the interaction between endothelial cells and breast cancer cells in vitro. We observed that CM from flow-stimulated osteocytes reduced endothelial permeability by 15% and breast cancer cell adhesion onto endothelial monolayers by 18%. The difference in adhesion was abolished with anti-intercellular adhesion molecule 1 (ICAM-1) neutralizing antibodies. Furthermore, CM from endothelial cells conditioned in CM from flow-stimulated osteocytes significantly altered the gene expression in bone-metastatic breast cancer cells, as shown by RNA sequencing. Specifically, breast cancer cell expression of matrix metallopeptidase 9 (MMP-9) was downregulated by 62%, and frizzled-4 (FZD4) by 61%, when the osteocytes were stimulated with flow. The invasion of these breast cancer cells across Matrigel was also reduced by 47%, and this difference was abolished by MMP-9 inhibitors. In conclusion, we demonstrated that flow-stimulated osteocytes downregulate the bone-metastatic potential of breast cancer cells by signaling through endothelial cells. This provides insights into the capability of bone mechanical regulation in preventing bone metastases; and may assist in prescribing exercise or bone-loading regimens to patients with breast cancers.

KEYWORDS

bone metastasis, breast cancer, endothelial cells, invasion, matrix metallopeptidase 9 (MMP-9), osteocyte, RNA sequencing

1 | INTRODUCTION

Recent advancements in cancer research have greatly improved treatments for breast cancer. The 5-year survival rate for localized breast cancer is now more than 90%.[1] However, bone metastasis, the spread of tumors to the bone, occurs in 65% to 75% of patients with advanced breast cancer[2] and remains difficult to treat. It is a severe complication that significantly worsens the bone quality of patients and has a 5-year survival rate of less than 15%.[3] Current therapies targeting bone metastasis, such as bisphosphonates and denosumab, may reduce pain and fracture, but do not cure bone metastasis or prolong survival.[4] Therefore, it is essential to establish an effective strategy in preventing bone metastasis before the secondary tumor is developed in the bone.

Dynamic loading on the bone is known to improve bone quality[5] and may mitigate bone metastasis. Although exercise is commonly suggested to patients with breast cancer to improve their quality of life,[6] patients suffering from bone metastasis often hesitate to follow due to concerns of increasing fracture risks. Furthermore, beacuase the incentive of improving quality of life is not strong, many patients with breast cancer may not be motivated enough to exercise. Yet, a recent clinical review revealed that exercise can in fact be beneficial for patients suffering from bone metastasis[7]; an in vivo study also showed that bone loading reduces the size of tumor and lesions created by the injection of breast cancer cells into the bone.[8] In addition, our previous in vitro study demonstrated that mechanical stimulation of cells in the bone can reduce the metastatic potential of breast cancer cells.[9] In this study, we intend to further investigate the mechanism of the preventative effect of mechanically stimulated bone cells on breast cancer bone metastasis.

One reason that breast cancers commonly metastasize to the bone is their ability to interact with the cells in the bone[10] and disrupt the bone remodeling balance. This not only causes bone lesions and increases fracture risk and pain, but also facilitates cancer cell migration and growth in the bone, thereby creating a vicious cycle between bone lesions and metastatic tumor growth. On the other hand, the bone remodeling balance is normally regulated by osteocytes, the major population of cells in the bone matrix.[11] Specifically targeting osteocytes' signaling to bone-resorbing osteoclasts reduced osteolysis during bone metastasis in vivo.[12] Importantly, the regulation of bone remodeling by osteocytes had also been shown to be mechanically stimulated.[13,14] Because the bone matrix is filled with interstitial fluid, an oscillatory fluid flow is created during bone-loading activities such as walking. A physiological level of shear stress of 0.8 to 3 Pa that results from this oscillatory fluid flow[15] had been shown to elicit response in osteocytes to signal to bone remodeling cells.[16-19] Consequently, mechanically stimulated osteocytes may break the vicious cycle of bone metastasis and be responsible for the attenuation of bone metastasis by bone loading.[8] Indeed, our previous in vitro study demonstrated that flow-stimulated osteocytes can reduce migration and increase apoptosis of breast cancer cells by signaling through osteoclasts or endothelial cells.[9]

Endothelial cells are present in large number and close proximity to the metastasizing cancer cells in blood vessels before a secondary tumor is established in the bone. Because the bone is highly vascularized, endothelial cells are ideally located to communicate with both the osteocytes in the bone matrix[20] and the metastasizing cancer cells in the blood stream. Endothelial cells had been extensively studied in the context of bone metastasis. In addition to being a passive barrier for the metastasizing cancer cells,[21] they also actively play a major role in interacting with the cancer cells.[22-24] Unfortunately, the drugs targeting endothelial cells, such as vascular endothelial growth factor (VEGF) inhibitors, have performed poorly in clinical trials.[25] Therefore, more investigation is needed. Previous research has shown that endothelial cells can respond to the factors secreted by osteocytes.[20,26] This suggests that endothelial cells may be able to relay signals from mechanically stimulated osteocytes to metastasizing cancer cells and downregulate metastasis.

Our preceding study demonstrated that signaling from flow-stimulated osteocytes to endothelial cells and breast cancer cells strongly reduced the transendothelial migration of breast cancer cells.[9] In this study, we aim to identify the mechanisms underlying this observation. Extravasation involves multiple processes. Factors that alter endothelial cells' permeability and adhesion molecule expression and breast cancer cells' ability to degrade the endothelial extracellular matrix have all been shown to affect extravasation.[27] Therefore, we investigated whether factors secreted by flow-stimulated osteocytes affect 1) endothelial permeability, 2) cancer cell adhesion onto endothelial monolayers, and 3) cancer cells' gene expression profile that may affect downstream invasiveness. This study presents the potential mechanisms for the regulation of cancer cells' metastatic potential by bone mechanical loading; and aims to offer novel insights into the importance of exercise for patients with breast cancer in preventing bone metastasis.

2 | MATERIALS AND METHODS

2.1 | Cell cultures

MLO-Y4 osteocyte-like cells (Gift from Dr Lynda Bonewald, Indiana University, Indianapolis, IN) were cultured in α-modified Eagle medium (catalog number 310-010-CL; Wisent, St-Bruno, QC, Canada) supplemented with 2.5%

fetal bovine serum (FBS; 12483-020; Gibco, Carlsbad, CA), 2.5% calf serum (CS) (16010-159; Gibco), and 1% penicillin-streptomycin (P/S) (15140122; Gibco), on petri dishes or glass slides coated with 0.15 mg/mL type-1 rat tail collagen (354236; Coring Life Science, Lowell, CA). Human umbilical vein endothelial cells (HUVECs; Gift from Dr Edmond Young, University of Toronto, Toronto, ON, Canada) were cultured in EndoMax medium (301-010-CL; Wisent) supplemented with 10% FBS and 1% P/S. Metastatic MDA-MB-231 human breast cancer cells (HTB-26; ATCC, Manassas, VA) were cultured in Kaighn's modification of Ham's F-12 medium (21127022; Gibco) supplemented with 10% FBS and 1% P/S. MDA-MB-231/1833 cells (Gift from Dr Arun Seth, Sunnybrook Research Institute, Toronto, ON, Canada), a subpopulation of MDA-MB-231 cells that had been selected in vivo to be specifically metastatic to the bone,[28] were cultured in Dulbecco modified Eagle medium (319-005-CL; Wisent) supplemented with 10% CS and 1% P/S. All cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

2.2 | Oscillatory fluid flow on osteocytes

Conditioned medium (CM) was acquired from MLO-Y4 osteocyte-like cells that were stimulated with oscillatory fluid flow, as described previously.[9] Briefly, parallel-plate flow chambers[29] and a linear actuator were used to apply oscillatory fluid flow at 1 Hz and 1 Pa peak shear stress to MLO-Y4 cells at 80% confluency for 2 hours. MLO-Y4 cells in the static-osteocyte groups were placed in the flow chambers, but not stimulated with flow; while the control MLO-Y4 cells were seeded on glass slides but not placed in flow chambers. MLO-Y4 cells were then incubated for 24 hours in 10 mL fresh medium before CM was obtained (osteocyte CM).

2.3 | Endothelial permeability

HUVECs were grown to full confluency on Transwells with a 0.4-μm pore-size semipermeable membrane (665641; Greiner Bio-One, Cassina de Pecchi, Italy) then conditioned in osteocyte CM for 6 hours. Two hundred microlitre of 1 mg/mL fluorescein isothiocyanate-dextran (FD40 [40-kDa fluorescent tracer]; Sigma-Aldrich, St. Louis, MI) was then added to the top of the Transwell and 500 μL phosphate buffered solution (PBS) was added to the bottom well. After 30 minutes, Transwells were removed and fluorescence was read for the bottom well.

2.4 | Adhesion on endothelial monolayer

HUVECs were grown to full confluency in the channels of μ-Slide VI 0.4 (ibidi, Martinsried, Bavaria, Germany), then conditioned in osteocyte CM for 16 hours. MDA-MB-231 breast cancer cells stained with cell tracker green (C2925; Life Technologies, Carlsbad, CA) were added to the channels with HUVECs at 800 000 cells/mL and allowed to adhere for 30 minutes. Oscillatory fluid flow was then applied to the channels with a linear actuator at 1 Hz and a 1 Pa peak shear stress for 30 minutes to wash away the loosely adhered MDA-MB-231 cells. The channels were then rinsed with PBS and the MDA-MB-231 cells that remained adhered were imaged and quantified with ImageJ (NIH, Bethesda, MD). Blocking experiments were performed as described previously[30] by adding 1 μg/mL mouse anti-human intercellular adhesion molecule 1 (ICAM-1) neutralizing antibody (BBA3; R&D Systems, Minneapolis, MN) to endothelial cells in osteocyte CM 1 hour before the addition of breast cancer cells.

2.5 | RNA sequencing

HUVECs grown to full confluency in six-well plates were conditioned in 50% osteocyte CM and 50% fresh HUVEC medium for 24 hours. The medium was then replaced with 100% fresh HUVEC medium to ensure that there was no leftover osteocyte-secreted factor, and the medium was collected from the conditioned HUVECs (endothelial CM) after 12 hours. MDA-MB-231/1833 bone-metastatic breast cancer cells grown to full confluency in six-well plates were subsequently conditioned in 50% endothelial CM and 50% fresh MDA-MB-231/1833 medium for 24 hours. RNA content was isolated from the conditioned MDA-MB-231/1833 cells with the RNeasy Mini Kit (74104; Qiagen, Venlo, Limburg, Netherlands), treated with the DNA-free Kit (AM1906; Life Technologies), and sent in for RNA sequencing at The Donnelly Sequencing Centre (University of Toronto). The library was prepared with the TruSeq Stranded mRNA Library Prep Kit (Illumina) and sequenced with NextSeq. 500 (Illumina; High output, 75 Cycles, v2 Chemistry; R1: 85 bp, IR1: 6 bp, single read). This sequencing data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7176.

Read counts were obtained with RNA Express v1.1 on Illumina's BaseSpace (https://basespace.illlumina.com), which aligns RNA sequencing reads to reference human genome hg38[31] using spliced transcripts alignment to a reference (STAR).[32] The differential gene expression was computed using DESeq2 v1.18.1[33] in R/Bioconductor.[34] Enriched gene ontology terms[35,36] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways[37-39] were computed on the significantly upregulated and downregulated genes[40] using GOseq v1.3[41] in R/Bioconductor

with count bias accounted for. Revigo[42] was used to trim down the list of gene ontology terms (allowed similarity = 0.5 using SimRel). The heat map was prepared with gplots 3.0.1[43] and the KEGG pathway map was prepared with pathview 1.18.2[44] in R/Bioconductor.

Quantitative polymerase chain reaction (qPCR) was done on selected genes to validate key results from RNA sequencing. The RNA contents isolated from conditioned MDA-MB-231/1833 cells, as described above, were treated with DNAse I (EN0521; Thermo Fisher Scientific, Waltham, MA) and reverse-transcribed using SuperScript III RT (18080-044; Invitrogen, Carlsbad, CA). qPCR was performed with LightCycler 480 SYBR Green I Master (04707516001; Roche, Mannheim, Baden-Württemberg, Germany) and gene-specific primers (listed in Table 1) purchased from Thermo Fisher Scientific (Waltham, MA).

2.6 | Invasion assay

One hundred microlitre of 1 mg/mL Matrigel (354234; BD Biosciences, Bedford, MA) was allowed to gel for 2 hours on a 24-well plate Transwells with an 8-μm pore-size semipermeable membrane (662638; Greiner Bio-One). MDA-MB-231/1833 bone-metastatic breast cancer cells conditioned in endothelial CM, as described above, were stained with cell tracker green. A total of 40 000 stained cells were added on top of each Matrigel-coated Transwell and allowed to invade towards 20%-FBS MDA-MB-231/1833 medium in the bottom wells for 24 hours. The cells were then fixed with 10% neutral buffered formalin. Non-invaded cells were scrapped off with cotton swabs and invaded cells were imaged and quantified with imageJ. Blocking experiments were performed by adding 1 μM matrix metallopeptidase 9 (MMP-9) inhibitor (ab142180; Abcam, Cambridge, Cambridgeshire, UK) to endothelial CM.

2.7 | Statistics

Each type of experiment was performed at least three times with each experiment containing at least three samples paired between the flow-osteocyte groups, where murine long bone osteocyte Y4 (MLO-Y4) osteocytes were stimulated with flow, and the static-osteocyte groups, where MLO-Y4 osteocytes were placed in flow chambers but not stimulated with flow. The paired samples were prepared as closely in time as possible, with the same reagents, and from the same dish of cells. The order within the pair was randomized to avoid time-dependent variations and this order was maintained throughout the sequential conditioning and experiments. The functional assay results were normalized to controls (results using CM from MLO-Y4 osteocytes seeded on glass slides but not placed in flow chambers) for each set of experiment to account for potential differences in experimental conditions. Student *t* test (two-tail and paired) was used to test significance between the static- and flow-osteocyte groups (significance was taken at $\alpha = 0.05$). For RNA sequencing results, a gene is considered differentially expressed when the adjusted *P* value (corrected for multiple testing with the Benjamini-Hochberg method) calculated by DESeq2 is less than 0.05.

3 | RESULTS

3.1 | Flow-stimulated osteocytes reduce endothelial permeability

First, we investigated whether factors secreted by flow-stimulated osteocytes affect the permeability of endothelial monolayers. To do this, CM was collected from flow-stimulated or static MLO-Y4 osteocytes. This CM (osteocyte CM) was added to monolayers of HUVECs. After 6 hours, endothelial permeability of this monolayer was measured using FD40 (40-kDa fluorescent tracer). Results in Figure 1 show that HUVECs conditioned in osteocyte CM from flow-stimulated MLO-Y4 osteocytes were 15% less permeable.

3.2 | Flow-stimulated osteocytes reduce cancer cell adhesion onto endothelial monolayer

Next, we investigated whether soluble factors from flow-stimulated osteocytes change endothelial monolayer's potential to be adhered by breast cancer cells. HUVEC monolayers were conditioned in osteocyte CM from either static of flow-stimulated MLO-Y4 osteocytes for 16 hours. MDA-MB-231 breast cancer cells were then allowed to adhere for 30 minutes before flow was applied to wash away the loosely adhered cells. We observed that

TABLE 1 Primers used for qPCR

Gene	Forward sequence (5′-3′)	Reverse sequence (3′-5′)
MMP-9	GAGGCGCTCATGTACCCTATGTAC	GTTCAGGGCGAGGACCATAGAG
FZD4	TTGGGCACGAGCTGCAGACG	TGAGCACACAGTTCAGGCTCCT
GAPDH	TTGCCATCAATGACCCCTTCA	CGCCCCACTTGATTTTGGA

Abbreviations: qPCR, quantitative polymerase chain reaction.

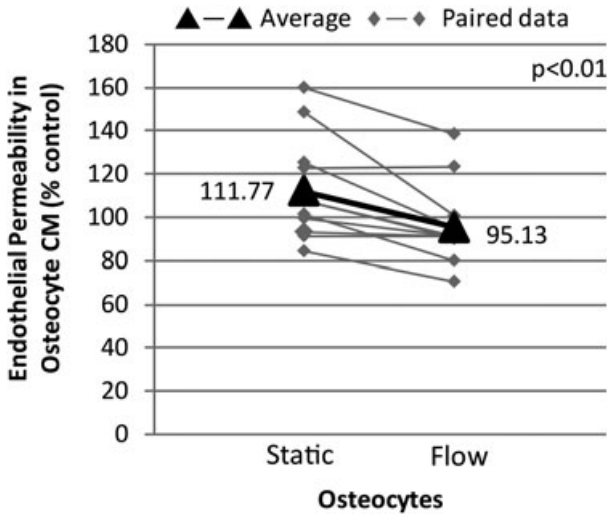

FIGURE 1 Endothelial permeability of HUVEC monolayers to 40-kDa fluorescent tracers (FD40) after 6-hour conditioning in CM from MLO-Y4 osteocytes that were static (but placed in flow chambers) or flow-stimulated, normalized to controls (HUVECs in CM from MLO-Y4 osteocytes not placed in flow chambers). Data are presented as paired data between static- and flow-osteocyte groups, n = 12 from three experiments. CM, conditioned medium; HUVECs, human umbilical vein endothelial cells; MLO-Y4, murine long bone osteocyte Y4

18% more MDA-MB-231 cells remained adhered when the HUVECs were conditioned in CM from static MLO-Y4 osteocytes (Figure 2).

Because ICAM-1 had been shown to be regulated by osteocyte CM[30] and is important for breast cancer cell adhesion to the endothelium,[45] extravasation,[46] and metastasis in vivo,[47] we hypothesized that it may be a key factor for the observed difference. Indeed, after incubating the conditioned HUVECs with anti-ICAM-1 neutralizing antibody, the difference in adhesion strength was abolished between the static- and flow-osteocyte groups (Figure 2).

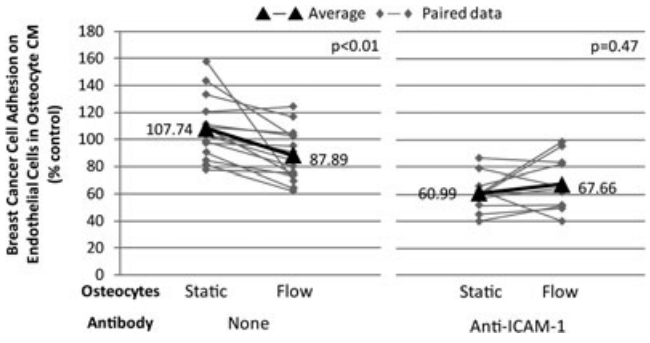

FIGURE 2 Adhesion strength of MDA-MB-231 breast cancer cells on endothelial monolayers of HUVECs after 16-hour conditioning in CM from MLO-Y4 osteocytes that were static (but placed in flow chambers) or flow-stimulated, normalized to controls (HUVECs conditioned in CM from MLO-Y4 osteocytes not placed in flow chambers), with and without anti-ICAM-1 neutralizing antibodies. Data are presented as paired data between the static- and flow-osteocyte groups, n = 15 from three experiments for no-antibody, n = 11 from three experiments for anti-ICAM-1. CM, conditioned medium; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; MLO-Y4, murine long bone osteocyte Y4

3.3 | Flow-stimulated osteocytes alter-cancer cell gene expression via endothelial cells

In addition to simply affecting endothelial cells, osteocytes may stimulate the endothelial cells to signal to the metastasizing breast cancer cells in blood vessels. Therefore, we used RNA sequencing to identify the differential gene expression between MDA-MB-231/1833 bone-metastatic breast cancer cells conditioned in endothelial CM from HUVECs conditioned in CM from static and flow-stimulated MLO-Y4 osteocytes. We used the 1833 bone-metastatic subpopulation of MDA-MB-231 breast cancer cells[28] for this part of the study because we were interested in genes that may be responsible for the tissue tropism. After sequencing and analyzing four pairs of RNA sequencing data with DESeq2 in R/Bioconductor,[33] we identified a list of significantly differentially expressed genes (adjusted P value < 0.05). Specifically, 15 genes (NEAT1, MMP-9, TFPI2, NNMT, LTBP3, EFEMP2, SAA1, SHE, FZD4, JAG2, RALA, BCL6B, PTGS2, GIMAP8, and SLC9B2) were significantly downregulated, and three genes (RPL28, C19orf53, and DPM2) were significantly upregulated, in MDA-MB-231/1833 bone-metastatic breast cancer cells when MLO-Y4 osteocytes were stimulated with flow (Figure 3). The top panel of Figure 3 shows the heat map for genes that were found to have significant, or a trend for (adjusted P value < 0.1), differential expression by the cancer cells between the static- and flow-osteocyte groups, along with their respective adjusted P values. Red indicates a high expression of the gene by breast cancer cells, while blue indicates a low expression.

Bottom panel of Figure 3 shows the gene ontology terms that involve the significantly differentially expressed genes. Gene ontology[35,36] and KEGG pathways [37-39] are two large databases compiled from past research to describe genes involved in cellular processes. We used the two databases to predict the differences in functions of cancer cells between the static- and flow-osteocyte groups. GOSeq in R/Bioconductor[41] was used to perform the enrichment analysis where the list of 18 significantly differentially expressed genes were mapped onto gene ontology terms or KEGG pathways. With this, 35 gene ontology terms were found to be downregulated (adjusted P value < 0.08), while none was upregulated, in

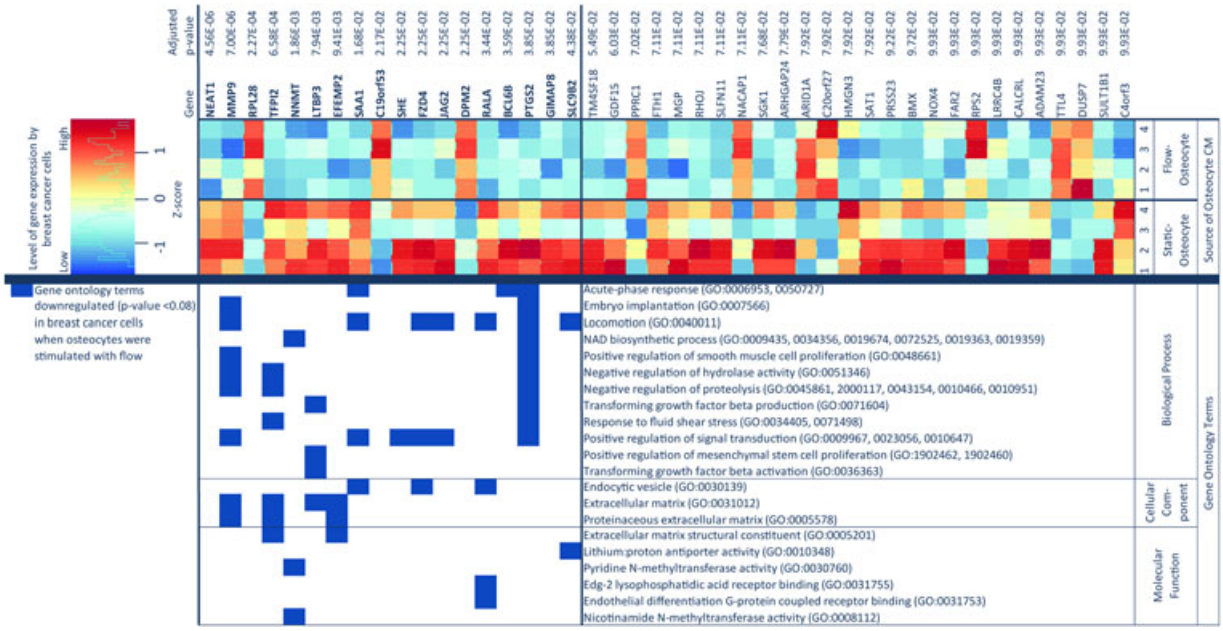

FIGURE 3 The differential gene expression between MDA-MB-231/1833 bone-metastatic breast cancer cells conditioned in endothelial CM from HUVECs conditioned in CM from MLO-Y4 osteocytes that were static (but placed in flow chambers) or flow-stimulated. Top panel shows the heat map (red: high expression; blue: low expression) of cancer cell gene expression with *P* values between the static- and flow-osteocyte groups (n = 4 paired samples), adjusted for multiple testing. Bottom panel shows the corresponding gene ontology terms that involve the significantly differentially expressed genes (blue: downregulated in the cancer cells of the flow-osteocyte group). CM, conditioned medium; HUVECs, human umbilical vein endothelial cells; MLO-Y4, murine long bone osteocyte Y4

MDA-MB-231/1833 bone-metastatic breast cancer cells when the MLO-Y4 osteocytes were stimulated with flow. Revigo[42] was subsequently used to combine similar gene ontology terms. In the bottom panel of Figure 3, blue squares represent gene ontology terms that were downregulated in the cancer cells when the osteocytes were stimulated with flow, shown under the corresponding genes. For example, the third row of the bottom panel in Figure 3 shows that locomotion of bone-metastatic breast cancer cells was reduced when the osteocytes were stimulated with flow because of the downregulation of MMP-9, SAA1, FZD4, JAG2, RALA, PTGS2, and SLC9B2. In addition, part of the cancer KEGG pathway (hsa05200) that contains the genes downregulated by breast cancer cells when osteocytes were stimulated with flow (MMP-9, FZD4, JAG2, RALA, and PTGS2; highlighted in blue) is shown in Figure 4.

3.4 | Flow-stimulated osteocytes reduce the cancer cell expression of MMP-9 and FZD4 via endothelial cells

Of the genes downregulated by MDA-MB-231/1833 bone-metastatic breast cancer cells when MLO-Y4 osteocytes were stimulated with flow, MMP-9 is of particular interest. It was the second most significantly differentially expressed gene from our RNA sequencing data, and is often implicated in metastasis for its importance in degrading the extracellular matrix.[48] It participates in several gene ontology terms and was the most significantly downregulated gene that is involved in the cancer KEGG pathway. SAA1 (serum amyloid A1) and FZD4 (frizzled 4), two other genes downregulated by cancer cells in the flow-osteocyte group, may also be of interest because serum amyloid A and interaction involving frizzled proteins had been shown to be upstream of matrix metalloproteinase production.[49,50] Because FZD4 is also involved in the KEGG cancer pathway, we performed qPCR on both MMP-9 and FZD4 as two key genes to verify our RNA sequencing results. Consistent with RNA sequencing results, we showed that MDA-MB-231/1833 breast cancer cells lowered their expression of MMP-9 by 62%, and FZD4 by 61%, when conditioned in CM from HUVECs conditioned in CM from flow-stimulated MLO-Y4 osteocytes (Figure 5).

3.5 | Flow-stimulated osteocytes reduce cancer cell invasiveness via endothelial cells by downregulating MMP-9

Because MMP-9 is a proteinase important for degrading the extracellular matrix,[48] we then performed an invasion assay to investigate whether the downregulation of

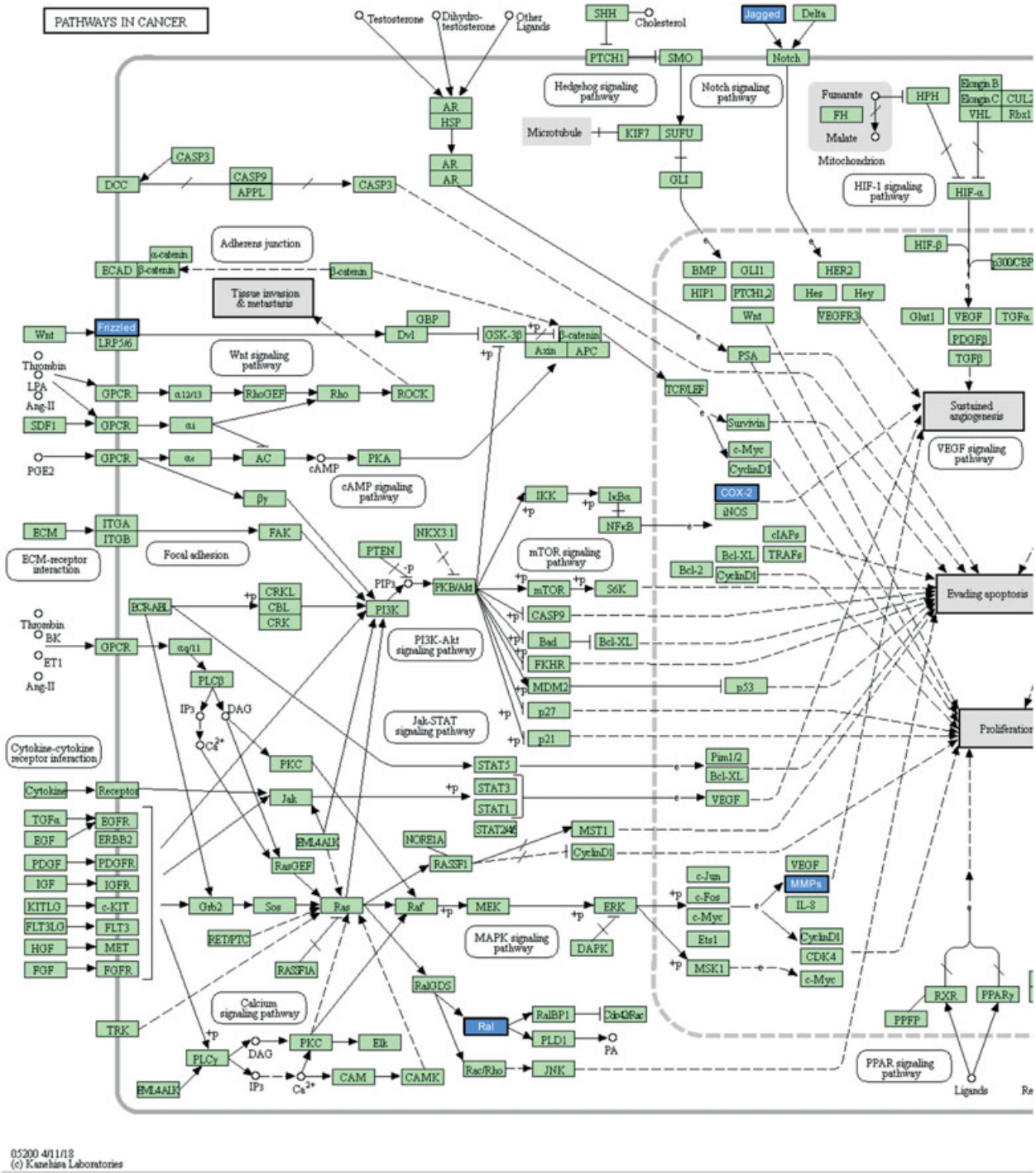

FIGURE 4 Part of the KEGG cancer pathway map, where green rectangles indicate all the genes involved in the pathway, and gray rectangles indicate downstream functions. Blue rectangles indicate the genes that were significantly downregulated in MDA-MB-231/1833 bone-metastatic breast cancer cells when conditioned in endothelial CM from HUVECs conditioned in CM from MLO-Y4 osteocytes stimulated with flow, in comparison to the results from the samples with static osteocytes that were placed in flow chambers.
CM, conditioned medium; HUVEC, human umbilical vein endothelial cell; KEGG, kyoto encyclopedia of genes and genomes; MLO-Y4, murine long bone osteocyte Y4

MMP-9 is relevant in terms of cellular function. Indeed, we found that the MDA-MB-231/1833 bone-metastatic breast cancer cells in the flow-osteocyte group, which are the cancer cells found to have 62% lower MMP-9 expression, had 47% less invasion across Matrigel-coated Transwells. This difference in invasion was abolished when MMP-9 inhibitor was applied (Figure 6), verifying the importance of MMP-9 in invasion.

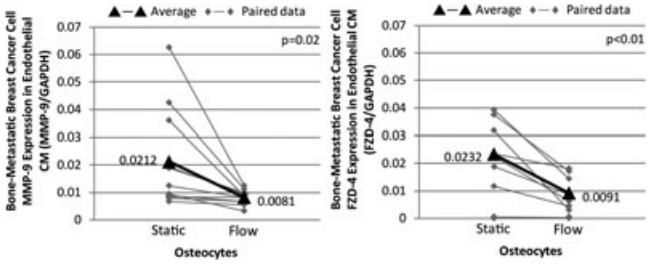

FIGURE 5 qPCR data showing the MMP-9 and FZD4 expression by MDA-MB-231/1833 bone-metastatic breast cancer cells conditioned in endothelial CM from HUVECs conditioned in CM from MLO-Y4 osteocytes that were static (but placed in flow chambers) or flow-stimulated, normalized to GAPDH expression. Data are presented as paired data between the static- and flow-osteocyte groups, n = 11 from three experiments. CM, conditioned medium; FZD4, frizzled 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; MLO-Y4, murine long bone osteocyte Y4; MMP-9, matrix metallopeptidase 9; qPCR, quantitative polymerase chain reaction

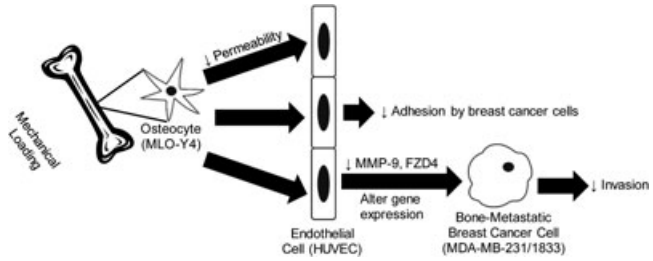

FIGURE 7 Summary of the effects of mechanically stimulated osteocytes on the interaction between endothelial cells and breast cancer cells to reduce the bone-metastatic potential. When the osteocytes are stimulated with oscillatory fluid flow, the application of osteocyte CM to endothelial cells reduces their permeability (Figure 1) and adhesion by breast cancer cells (Figure 2). The application of CM from these conditioned endothelial cells to bone-metastatic breast cancer cells reduces their MMP-9 and FZD4 expression (Figures 3 and 5) and invasiveness (Figure 6). CM, conditioned medium; FZD4, frizzled 4; MMP-9, matrix metallopeptidase 9

4 | DISCUSSION

Bone mechanical loading has been shown to have downregulatory effects on bone metastasis.[8] Our previous in vitro study identified signaling from osteocytes through endothelial cells as a potential pathway for these observations.[9] In this study, we identified the elements in the extravasation cascade that are affected by molecules released from osteocytes stimulated with oscillatory fluid flow (2 hours at 1 Hz and a peak shear stress of 1 Pa). We found that flow-stimulated osteocytes reduced endothelial cells' permeability and ability to be adhered by breast cancer cells, as well as strongly reduced the invasive potential of breast cancer cells through signaling mediated by endothelial cells (Figure 7). RNA sequencing was used to identify genes significantly altered in bone-metastatic breast cancer cells.

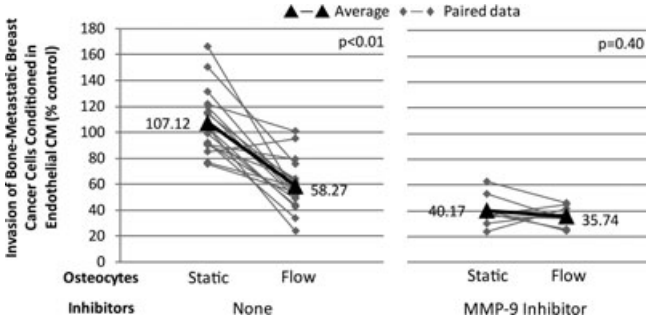

FIGURE 6 Invasion through Matrigel-coated Transwells by MDA-MB-231/1833 bone-metastatic breast cancer cells after conditioning in endothelial CM from HUVECs conditioned in CM from MLO-Y4 osteocytes that were static (but placed in flow chambers) or flow-stimulated, normalized to controls (invasion of MDA-MB-231/1833 cells when MLO-Y4 cells were not placed in flow chambers). Data are presented as paired data between the static- and flow-osteocyte groups, n = 18 from three experiments for no-inhibitor, n = 9 from two experiments for MMP-9 inhibitor. CM, conditioned medium; HUVECs, human umbilical vein endothelial cells; MLO-Y4, murine long bone osteocyte Y4; MMP-9, matrix metallopeptidase 9

We first observed that endothelial monolayers conditioned in CM from flow-stimulated osteocytes had a slightly reduced permeability to FD40 (Figure 1). This may be becuase of the change in the expression of several factors secreted by osteocytes in response to flow. For example, the increased production of prostaglandin E2,[51] nitric oxide,[52] adenosine triphosphate,[53] and sphingosine-1-phosphate[54] by flow-stimulated osteocytes may contribute in making the endothelial layer less permeable. These factors have been shown to maintain the endothelial layer by enhancing either the adheren junctions or the glycocalyx layer.[55-58] As cancer cell extravasation requires the degradation of glycocalyx and the separation of endothelial cells,[59] the increased production of these factors will reduce extravasation. This aligns well with our previous study showing reduced extravasation towards CM from flow-stimulated osteocytes.

We then observed that the adhesion strength of breast cancer cells to endothelial monolayers was attenuated when the endothelial cells were incubated in CM from flow-stimulated osteocytes (Figure 2). Upregulations of E-selectin and ICAM-1 by endothelial cells have been shown to be responsible for increasing breast cancer cell adhesion to the endothelium.[60] However, the induced upregulation of selectins returns to basal levels after 6 to 12 hours,[46] which is shorter than the 16-hour conditioning of endothelial cells in our experiment. Therefore, we hypothesized that the

difference we observed is becasue of the difference in the ICAM-1 expression, and not the selectin expression. Indeed, the difference in adhesion strength was abolished after blocking with anti-ICAM-1 neutralizing antibodies (Figure 2). Although adhesion was reduced in the flow-stimulated osteocyte group as well, it was not completely abolished in either group. This suggests that ICAM-1 is responsible for the difference in breast cancer cell adhesion to endothelial monolayers between the static- and flow-osteocyte groups. The ICAM-1 expression is inducible by shear stress and a variety of cytokines. A previous study had shown the regulation of the endothelial ICAM-1 expression by osteocyte-secreted interleukin-6,[30] which may be responsible for our observed phenomenon.

We then performed a double-conditioning experiment to explore effects on breast cancer cells, where endothelial cells were first conditioned in CM from static or flow-stimulated osteocytes, and bone-metastatic breast cancer cells were then cultured in CM collected from these endothelial cells. Using CM instead of a direct co-culture with osteocytes and endothelial cells guarantees that only soluble factors were involved. CM from osteocytes was also replaced with fresh medium 12 hours before the collection of CM from endothelial cells to ensure that the collected endothelial CM did not contain factors secreted by osteocytes. This not only narrows down the potential factors, but also better mimics the physiological environment because it is more likely for the osteocytes in the bone matrix to signal to the blood vessel via secreted factors. The MDA-MB-231/1833 bone-metastatic subpopulation of MDA-MB-231 breast cancer cells was used for this experiment because we were interested in potential changes in the genes that allow for the bone-metastatic specificity.

With the double-conditioning experiment, we observed 18 significantly differentially expressed genes in the bone-metastatic breast cancer cells when the osteocytes were stimulated with oscillatory fluid flow in comparison to static-osteocyte groups (Figure 3). The adjusted *P* values calculated by DESEq2 were selected to rank the differentially expressed genes because the *P* values consider both the fold change and the expression level of a gene and, therefore, excludes genes with low fold-changes as well as false-positives with large fold-changes but low expressions or high variations. The differentially expressed genes were then used to identify significantly affected gene ontology terms and KEGG pathways. Although no gene ontology terms were found to be significantly enriched after Benjamini-Hochberg correction, 35 gene ontology terms exhibited trends of being downregulated ($P < 0.08$) in breast cancer cells when osteocytes were stimulated with flow. This includes processes important in metastasis, such as cellular response and signaling (MMP-9, SAA1, SHE, FZD4, JAG2, BCL6B, and PTGS2), locomotion (MMP-9, SAA1, FZD4, JAG2, RALA, PTGS2, and SCL9B2), TGF-β (transforming growth factor beta) production and activation (LTBP3, PTGS2, and EFEMP2), and extracellular matrix (MMP-9, TFPI2, LTBP3, and SAA1). Moreover, genes MMP-9, FZD4, JAG2, RALA, and PTGS2, which were downregulated in bone-metastatic breast cancer cells when osteocytes were stimulated with flow, participate in the KEGG cancer pathway (Figure 4). Furthermore, although the most significantly downregulated gene, NEAT1, is not involved in any significantly enriched gene ontology terms or the KEGG cancer pathway, it is a nuclear long non-coding RNA that has been shown to be correlated with poor survival in patients with breast cancer.[61]

We also examined the sequencing data further for variations. First, we verified that the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the housekeeping gene used for normalization in qPCR, was constant across samples, with a fold change of 1.004 and an adjusted *P* value of 1.000 between static- and flow-osteocyte groups. This also ensures that the observed differences in the gene expressions were not because of the variation in cell number. However, the heat map shown in Figure 3 makes it apparent that breast cancer cells in sample 3 of the static-osteocyte group had an abnormally low expression for most genes. Therefore, we repeated the differential gene expression analysis excluding the paired sample three from static- and flow-osteocyte groups to verify that the overall trend was not affected by this outlier. After doing this, we saw that 174 genes were downregulated, and only 1 gene (RPL28) was upregulated, in the bone-metastatic breast cancer cells when the osteocytes were stimulated with flow. Although *P* values changed after the exclusion of sample three, all the genes that were found to be downregulated in the cancer cells of the flow-osteocyte group remained to be so. Intriguingly, C19orf53 and DPM2 were no longer significantly upregulated after the removal of sample three. This is probably becasue of their abnormally high expression in sample three of the flow-osteocyte group.

Of the 18 genes differentially expressed by bone-metastatic breast cancer cells when conditioned in CM from endothelial cells conditioned in CM from flow-stimulated osteocytes, the downregulation of MMP-9 was especially worth noting. MMP-9 is often implicated in metastasis as it plays a major role in extracellular matrix degradation and extravasation.[48] From Figure 3 and Figure 4, it is apparent that MMP-9 participates in many gene ontology terms, as well as the KEGG cancer pathway. Therefore, we performed qPCR on MMP-9 and FZD4, a potential upstream promoter of MMP-9[50] that is also involved in the KEGG cancer pathway, and verified that they were significantly downregulated (Figure 5). Our invasion assay results also confirmed that cancer cells in conditions found to have the lower MMP-9 expressions also had reduced invasion through Matrigel-coated Transwells (Figure 6). This is relevant

because the invasiveness of breast cancer cells affects their invasion through the basement membrane of endothelial cells and the bone in vivo. Furthermore, the application of MMP-9 inhibitors abolished the difference in invasion, while invasion was not completely blocked. This suggests that signaling from flow-stimulated osteocytes through endothelial cells reduced the invasive potential of breast cancer cells by downregulating MMP-9, which may also involve the downregulation of potential upstream genes such as FZD4[50] and SAA1.[49] In addition to these genes, PTGS2 (prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase-2), another significantly downregulated gene, plays a role in many biological processes, such as locomotion and TGF-β production. It may be of interest as well because cell locomotion is essential for metastasis and TGF-β is often implicated in metastasis.[62]

In conclusion, we observed that flow-stimulated osteocytes reduced the endothelial permeability and the adhesion of breast cancer cells on the endothelial monolayers. Interestingly, we showed that the gene expression of breast cancer cells can be strongly regulated by signaling from flow-stimulated osteocyte through endothelial cells. Specifically, we demonstrated the reduction in the invasiveness of bone-metastatic breast cancer cells through the downregulation of MMP-9 and FZD4, two genes that play a major role in cancer and metastasis, when osteocytes were stimulated with flow. This study further explored the downregulation of the bone-metastatic potential by mechanically stimulating the cells in the bone; and suggests that bone-loading exercise may prevent bone metastasis.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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