# Mechanical Regulation of Breast Cancer Bone Metastases via Direct and Indirect Osteocyte Signaling

Yu-Heng Vivian Ma[1], Candy Lam[1], Shreyash Dalmia[1], Peter Gao[1], Jacob Young[1], Chao Liu[1], Kevin Middleton[1], Lidan You[1,2]
[1]Institute of Biomaterials and Biomedical Engineering, [2]Department of Mechanical and Industrial Engineering
University of Toronto, Toronto, ON, Canada

## INTRODUCTION

Bone Metastases (migration of cancers to bone)

- Common and severe complications of cancer with an estimate of 65-80% of patients with advance breast cancer developing bone metastasis [1].
- Symptoms include pathological bone fractures, pain, hypercalcaemia, spinal cord and nerve-compression syndromes.
- Metastasized cancer cells are capable of **disturbing the normal bone turnover balance**, causing bone lesions and accelerating the release of growth factors from bone matrix, which in turn aid cancer cell survival.

Osteocytes

- Major population of cells in the bone.
- **Mechanosensors** of the bone that translate mechanical loading on the bone (experienced by osteocytes as oscillatory fluid flow through the lacunae-canalicular network in which they reside) into signals to other cells (such as bone-forming osteoblasts and bone-resorbing osteoclasts) and **regulate bone turnover**.
- Therefore, exercise, often suggested as an intervention for patients suffering from breast cancer, regulate bone remodeling via osteocytes.

Osteocytes and Bone Metastasis

- Limited knowledge on the effect of bone mechanical loading on the progress of bone metastasis.
- *In vivo* study showed that mechanical loading through tibial compression reduces the growth of secondary breast tumors after they metastasized to the bone [2]. However, the mechanism and the preventative potential of exercise is not discussed.
- Osteocytes stimulated with uni-directional flow increased connexin hemichannels expression and ATP release, which reduced breast cancer cell migration [3].
- Conditioned media from osteocytes stimulated breast cancer cell proliferation and migration [4].

## HYPOTHESIS

**Mechanically stimulated osteocytes will affect bone metastasis by signalling to breast cancer cells directly as well as indirectly through other cells in the bone**

## METHODS

Cell Culture

- MLO-Y4 osteocyte-like cells (gift of Dr. Bonewald, Indiana University): Cultured in α-MEM (2.5% CS, 2.5% FBS, 1%PS) on collagen-coated surface.
- RAW264.7 osteoclast precursors: Cultured in DMEM (10% FBS, 1% PS, 2% L-glutamine).
- Human Umbilical Vein Endothelial Cells (HUVECs) (gift of Dr. Young, University of Toronto): Cultured in endothelial cell growth media from Lonza.
- MDA-MB-231 highly metastatic breast cancer cells: Cultured in F-12K media (10% FBS, 1%PS).
- MCF-7 less-metastatic breast cancer cells: Cultured in Eagle's MEM (10% FBS).

Mechanical Loading through Oscillatory Fluid Flow

- Parallel-plate flow chamber
- Sinusoidal wave: 1 Pa peak shear stress, 1Hz, 2 hours
- Cells at 80% confluency at the beginning of flow.
- Conditioned media (CM) collected 24 hours post-flow.

Cancer Cell Activity Assays

- Migration: Cell-tracker-green-stained cancer cells were placed on Transwell and allowed to migrate towards CM for 6 hours. Cells that did not migrate were scraped off and migrated cells were counted.
- Trans-endothelial migration: 18-hour migration with a layer of HUVECs seeded on collagen-coated Transwells.
- Apoptosis: APOPercentage
- Viability: Fixable Viability Dye eFluor 450, flow cytometry.
- Proliferation: BrdU, flow cytometry

Osteoclastogenesis

- RAW264.7 cells were cultured in 50% CM supplemented with 10ng/mL RANKL (receptor activator of nuclear factor kappa-B ligand; essential for osteoclastogenesis). Medium was replaced with 100% RAW264.7 growth media on day 6.
- CM was collected and cells were stained for TRAP (tartrate-resistant acid phosphatase; highly expressed by osteoclasts) on day 7. Osteoclasts are identified as TRAP-positive multi-nucleated cells.

Statistics

- P-values were obtained using paired Student's t-test.
- Data normalized to MLO-Y4 cell count and controls (results of MDA-MB-231 in CM from MLO-Y4 cells not placed in flow chambers).

## RESULTS

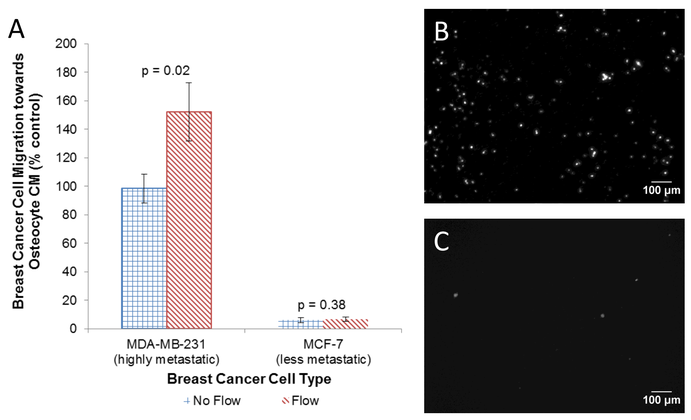



**Fig. 1. (A)** CM from flow-stimulated MLO-Y4 osteocytes increased migration of MDA-MB-231 breast cancer cells. MCF-7 cells did not migrate towards MLO-Y4 CM. Data is presented as mean ± standard deviation, n = 14 from 3 experiments. Sample images of cell-tracker-stained **(B)** MDA-MB-231 and **(C)** MCF-7 Transwell migration are presented.

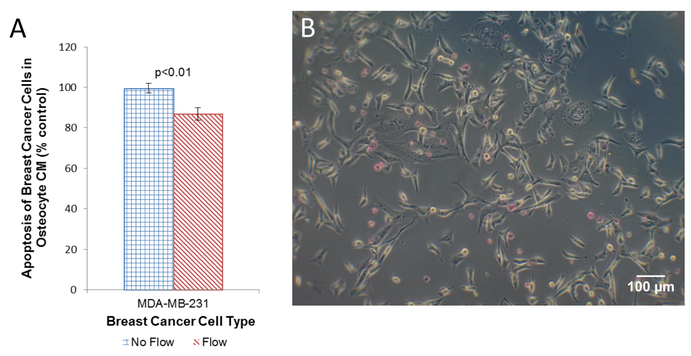



**Fig. 2 (A)** Apoptosis of MDA-MB-231 breast cancer cells is slightly lower in CM from flow-stimulated MLO-Y4 osteocytes. Data is presented as mean ± standard deviation, n = 8 from 2 experiments. **(B)** Sample image of MDA-MB-231 cells stained with APOPercentage. Pink cells indicate cells undergoing apoptosis. No difference was found for cancer cells viability and proliferation (not reported).

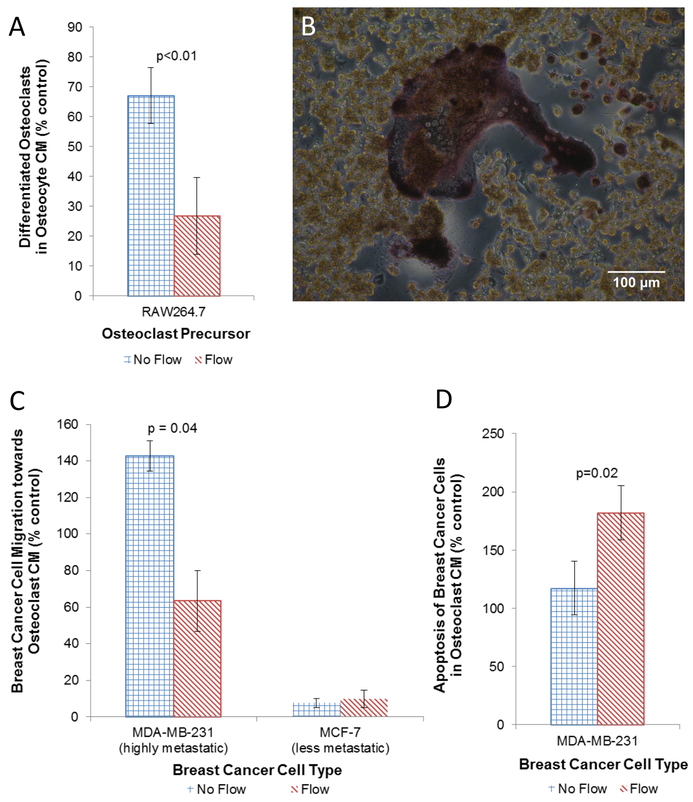



**Fig. 3. (A)** CM from flow-stimulated MLO-Y4 osteocytes reduced osteoclastogenesis. **(B)** Sample image of differentiated osteoclasts. CM from RAW264.7 osteoclasts cultured in CM from flow-stimulated osteocytes **(C)** reduced migration and **(D)** increased apoptosis of MDA-MB-231 breast cancer cells. MCF-7 cells did not migrate towards CM. Data is presented as mean ± standard deviation, n = 29 from 4 experiments.

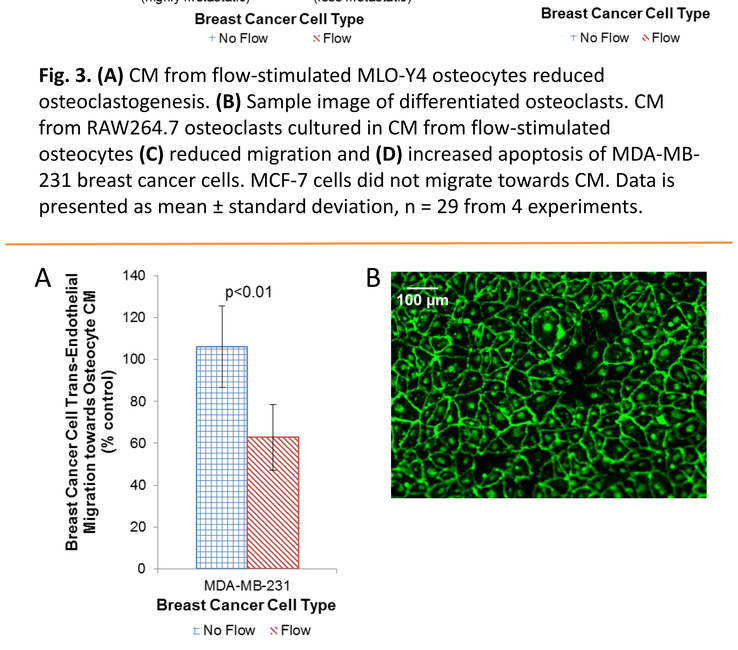



**Fig. 4 (A)** CM from flow-stimulated MLO-Y4 osteocytes increased trans-endothelial migration of MDA-MB-231 breast cancer cells. Data is presented as mean ± standard deviation, n = 23 from 4 experiments. **(B)** Sample image of HUVECs on Transwells stained for VE-Cadeherin.

## SUMMARY

Osteocyte → Breast Cancer Cells (Direct Signaling)

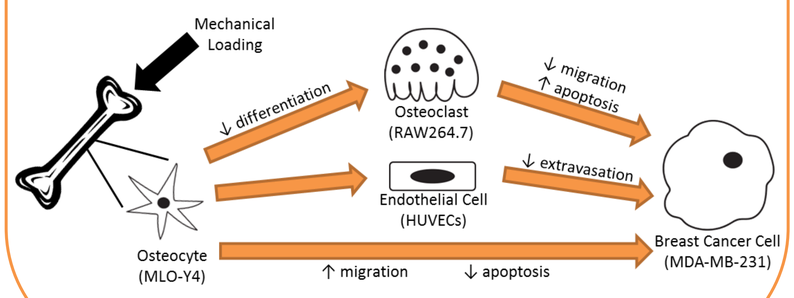
Conditioned media (CM) from MLO-Y4 osteocytes stimulated with 2-hour oscillatory fluid flow increased migration (Fig. 1) and slightly reduced apoptosis (Fig. 2) of MDA-MB-231 breast cancer cells. Less metastatic MCF-7 breast cancer cells did not migrate towards CM.

Osteocyte → Osteoclast → Breast Cancer Cells

CM from flow-stimulated osteocytes resulted in lower osteoclastogenesis (Fig. 3A). CM from this group (with fewer osteoclasts) reduced migration (Fig. 3C) and increased apoptosis (Fig. 3D) of MDA-MB-231 cells. MCF-7 cells did not migrate towards CM.

Osteocyte → Endothelial Cells → Breast Cancer Cells

CM from flow-stimulated osteocytes reduced trans-endothelial migration of MDA-MB-231 cells (Fig. 4A).




## DISCUSSION

- Direct and indirect signaling from mechanically stimulated osteocytes seem to have opposite effects on bone metastasis.
- The increased MDA-MB-231 migration towards flow-stimulated MLO-Y4 cells may be due to the increased VEGF secretion by osteocytes under mechanical loading (data not shown). VEGF has been shown to increase CXCR-4 expression of breast cancer cells and thus their migration towards SDF-1 in media [5]. Applying anti-VEGF neutralizing antibody abolished the observed preferential migration (data not shown). The difference between results observed in this study and [3] may have resulted from our 24-hour post-flow incubation since ATPs degrade within minutes outside of cells [6].
- Mechanically stimulated osteocytes reduce osteoclastogenesis and, therefore, osteoclasts' support of cancer cell growth and migration [7]. S1P is a potential target since it is secreted by osteoclasts [8] and is shown to support cancer cell migration and growth [9].
- Osteocytes that are stimulated with flow change their expression of various factors [10-13] that could affect endothelial permeability [14-17] and subsequent cancer cell extravasation.
- Future work: Micro-channel device may be used to allow real-time cross-talk between different cell populations. This better mimics the *in vivo* bone environment, while excluding interferences and variations that exist *in vivo*. Mechanisms will be investigated. Animal studies will also be performed to verify the *in vitro* findings.
- Significance: Provides insights into the effect of exercises on bone metastases, and assists in designing breast cancer intervention programs that also lowers the risk for bone metastases.

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## ACKNOWLEDGEMENTS

This research is supported by the National Sciences and Engineering Research Council (NSERC) , Canadian Institutes of Health Research (CIHR), and Ontario Graduate Scholarship (OGS)