Reduction of breast cancer extravasation via vibration activated osteocyte regulation

Xin Song, Chun-Yu Lin, Xueting Mei, Liyun Wang, Lidan You
youlidan@mie.utoronto.ca

Highlights
LMHF vibration reduced breast cancer extravasation via osteocyte regulation
Piezo1 knockdown impaired osteocyte response to vibration
Chemical activation of Piezo1 further reduced cancer extravasation under vibration

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SUMMARY

Physical exercise benefits breast cancer patients by reducing cancer progression and promoting bone health. However, intense exercise is physically challenging for bedridden, disabled, or aged patients. As an exercise surrogate, low-magnitude (<1 g) high-frequency (>30 Hz) (LMHF) vibration has gained growing interest in recent years, but its effects on bone metastasis remain unknown. We used a microfluidic co-culture platform that mimics bone-cancer environments to study the impact of vibration on breast cancer extravasation. LMHF vibration activated osteocytes, the primary mechanosensing cells in bones, which reduced cancer extravasation by 43%. We further studied the vibration mechanism by demonstrating the important role of the Piezo1 ion channel in osteocyte mechanotransduction. Chemical activation of Piezo1 enhanced osteocyte inhibition of cancer extravasation under vibration at the early time point. These data indicated that LMHF vibration could inhibit cancer extravasation, suggesting that vibration may suppress bone metastasis in breast cancer patients.

INTRODUCTION

Most cancer-associated deaths are because cancer spreads to other parts of the body through metastasis. Bones are one of the most common sites of metastasis, which occurs in 75% of patients with advanced breast cancers.1 During metastasis, cancer cells first cross the endothelial barrier into the bloodstream (intravasation) and travel through the blood vessels as circulating tumor cells (circulation); they then cross the endothelial barrier again to reach bones (extravasation), in which they proliferate and establish colonies (colonization).2 Metastasized cancer cells disrupt the balance between “bone-resorbing” osteoclasts and “bone-forming” osteoblasts, which is primarily maintained by osteocytes, leading to incurable bone lesions.3 Current standard treatments such as surgery, radiation, and chemotherapy only can slow cancer progression and have serious side effects.4 Therefore, developing new interventions to prevent bone metastasis is becoming increasingly important.

We investigated the potential of harnessing bone’s innate anabolic response to mechanical stimulation to prevent bone metastasis. Physical exercise was shown to decrease the risk of skeletal-related events (SREs) for breast cancer patients.5 In vivo animal studies indicated that mechanical loading during exercise reduced the metastatic potential of breast cancers and suppressed bone destruction caused by cancer metastasis.6,7 However, for bedridden, disabled, or aged patients, intensive physical exercises are challenging or even can cause unintended injuries. In search of a safer alternative, low-magnitude (<1 g) high-frequency (>30 Hz) (LMHF) vibration has gained attention recently due to its nature of being effective, easy to perform, and safe. Several studies reported that LMHF vibration improved bone mineral density (BMD) and enhanced bone strength for young childhood cancer survivors8 and postmenopausal women younger than 65 years.9–11 In animal models, vibration increased BMD12–15 and promoted fracture healing.16 Therefore, LMHF vibration, which has been shown to promote bone health, could serve as an exercise alternative.

Nevertheless, recent clinical studies indicated that LMHF vibration had minimum or no beneficial effects for the elderly (>65 years old), accounting for about 50% of total breast cancer patients.9,17,18 Despite the good efficacy on BMD improvement and bone fracture healing, vibration did not demonstrate similar effects in aged animal models.16,19 Age-related degeneration of the intricate lacunocanalicular network (LCN) may lead to altered LCN architecture, impaired fluid dynamics, and reduced mechanosensitivity in bones.20–22 To overcome the mechanosensing barrier for the elderly, we focus on Piezo1, an ion channel highly expressed in bones. Recent animal studies demonstrated the importance of Piezo1 that mice with Piezo1
knockout in osteocytes and osteoblasts were generally small and weak, and loss of Piezo1 also blunted their skeletal response to mechanical loading. On the other hand, chemical activation of Piezo1 by Yoda1 increased the intracellular calcium concentration and stimulated mechanosensitive gene expressions. Yoda1 injection into mice could increase bone mass by increasing cortical thickness in the vertebra. Due to Piezo1’s important role in mechanotransduction, we believe that Yoda1 can potentially elevate vibration effects. The mechanical stimuli applied to the whole body are transduced to the organ level, tissue level, and finally, the cellular level. Osteocytes are the major mechanosensors and regulators, comprising over 90% of the total bone cells. They sense mechanical stimuli through integrins and mechanosensitive ion channels (e.g., Piezo1). Then they transduce these mechanical signals into biochemical signals through different pathways involving intracellular Ca²⁺, nitrogen oxide (NO), prostaglandins (PGE2), Wnts, and adenosine triphosphates (ATPs). Osteocytes secrete downstream effectors such as receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), and sclerostin (Sost), which affect osteoclast and osteoblast activities. Other than osteoclasts and osteoblasts, osteocytes have recently been investigated for their roles in regulating cancer behaviors in response to fluid shear stress, mimicking interstitial fluid flow from bone loading during exercise. In general, conditioned media, which contains all soluble factors secreted from flow-stimulated osteocytes, directly promoted breast cancer progression. This direct osteocyte-cancer interaction is more common at the later stage of bone metastasis when osteocytes are closely in contact with cancer cells after cancer colonization. At the early stage of bone metastasis, which our research focuses on, osteocytes reside inside bones and do not physically interact with circulating cancer cells in the blood vessels. For extravasation to occur, the cancer cells need to cross the endothelial barrier before reaching bones. Therefore, we speculate that osteocytes communicate with cancer cells via endothelial cell signaling at this stage. Evidence showed that conditioned media collected from flow-stimulated osteocytes indirectly reduced breast cancer progression through endothelial cells. Using our novel microfluidic platform, we showed that flow-stimulated osteocytes decreased transendothelial breast cancer invasion (extravasation), suggesting that osteocytes play a crucial role in cancer extravasation, and exercise may help inhibit this process.

To date, few studies have investigated the osteocyte regulation of bone metastasis under LMHF vibration. Most vibration studies focus on breast cancer alone. Although cancer cells can respond to mechanical stimulation, osteocytes are considered the primary mechanosensing cells in bones; hence these studies should include osteocytes. In addition, bone metastasis involves multiple types of cells, including cancer cells, endothelial cells, and bone cells. Therefore, targeting the cellular crosstalk in the bone-cancer environment is more constructive than targeting cancer cells alone. Our just-published conditioned medium study provided evidence that vibration-activated osteocytes reduced osteoclast formation and breast cancer migration with the help of Yoda1. Nevertheless, conventional cell cultures or conditioned medium studies do not allow for real-time cell-cell communication. Some in vitro studies developed microfluidic platforms which successfully mimicked bone-cancer environments. They established bone extracellular matrix secreted directly from 14-day differentiated hBM-MSCs and regenerated microvascular network for cancer extravasation using human umbilical vein endothelial cells (HUVECs). However, neither mechanical stimulation nor osteocytes were included.

In this study, we utilized a microfluidic co-culture platform capable of resembling bone-cancer environments and integrating mechanical stimulation to study vibration effects on breast cancer extravasation. We hypothesize that LMHF vibration activates osteocytes, leading to cellular crosstalk among osteocytes, endothelial cells, and cancer cells that reduces breast cancer extravasation. In addition, we investigated the role of Piezo1 in osteocyte response to vibration. We further studied if chemically activating Piezo1 by Yoda1 could enhance the vibration effect. This is the first research that targets osteocyte-cancer interactions under LMHF vibration using a physiologically relevant tissue model, which is an essential step toward developing a safe treatment for the high-risk population.

RESULTS

Vibration-activated osteocytes by regulating the expressions of mechanosensitive genes

LMHF vibration (60 Hz, 0.3 g, 1 h, Figure 1) significantly up-regulated the expressions of Piezo1 (1.63-fold) and COX-2 (1.32-fold) and down-regulated the expression of RANKL (0.86-fold). The overall RANKL/OPG (0.62-fold) was reduced after vibration stimulation (Figure 2).
Piezo1 knockdown impaired osteocyte response to vibration

Piezo1 siRNA transfection achieved 78% of Piezo1 knockdown in MLO-Y4 osteocytes compared to those transfected with negative control siRNAs (Figure 3A). To identify Piezo1 roles under vibration, we quantified mRNA expressions of mechanosensitive genes in osteocytes transfected with Piezo1 or negative control siRNAs under static or vibration conditions. Vibration (60 Hz, 0.3 g, 1 h) increased the expressions of Piezo1 (2.32-fold), COX-2 (3.17-fold), RANKL (2.41-fold), and OPG (2.97-fold) in osteocytes transfected with negative control siRNAs. With siRNA-mediated knockdown of Piezo1, vibration still up-regulated the expression of COX-2 (1.35-fold). However, Piezo1 knockdown blunted vibration stimulation of Piezo1, COX-2, OPG, and RANKL (Figure 3B).

Vibration had no effects on cell growth and viability in devices

We employed our custom-made microfluidic platform integrating with vibration stimulation (Figure 4). We quantified total cell numbers and live/total proportions of osteocytes, endothelial cells, and breast cancer cells seeded in the platform. Vibration (60 Hz, 0.3 g, 1 h/day for 3 days) did not significantly impact cell growth and viability (Figure 5).

Vibration reduced breast cancer invasion via direct and indirect osteocyte signaling

We measured the direct effect of LMHF vibration on cancer invasion. We observed that vibration (60 Hz, 0.3 g, 1 h/day for 3 days) on breast cancer cells alone (i.e., only seeding cancer cells in the lumen channel) did not affect its invasion (Figure 6A).

We then investigated the direct osteocyte regulation of breast cancer invasion. Breast cancer cells in the lumen channel were co-cultured with osteocytes in the adjacent channel. We observed that vibration decreased cancer invasion distance by 24% (Figure 6B). This direct signaling between osteocytes and cancer cells is more relevant to post-colonization in vivo.

To better mimic the cancer extravasation (i.e., transendothelial cancer invasion) in vivo, we seeded a circular layer of HUVEC endothelial cells in the lumen channel. We validated this HUVEC-coated hydrogel lumen in our previous study. In specific, HUVECs produced VE-cadherin intercellular connections, and the HUVEC-coated hydrogel allowed for the exchange of regulatory signals.35 We observed that cancer extravasation distance was reduced by 43% in the LMHF vibration condition compared to that in static (Figure 6C). To investigate whether the observed decrease was due to osteocyte regulation, we quantified the cancer extravasation distance with and without osteocytes under vibration. We observed that the cancer extravasation distance was reduced by 52% with osteocytes compared to without (Figure 6C).

Piezo1 activation in osteocytes facilitated their regulation of cancer extravasation at the early time point

Chemical activation of Piezo1 by Yoda1 (10 μM, 2 h/day) in the osteocyte channel accelerated the vibration (60 Hz, 0.3 g, 1 h/day) effect that decreased breast cancer extravasation by 25% compared to that in DMSO...
control after one-time treatment (Yoda1+LMHF vs. DMSO on Day 2, Figure 7B). After three-time treatments (on Day 4), vibration alone reduced breast cancer extravasation, consistent with the results observed in Figure 6C. While the activation of Piezo1 using Yoda1 did not further enhance the vibration effect on Day 4 (Figure 7B).

DISCUSSION

Bone metastasis is a common and severe complication that is the major cause of breast cancer deaths. To suppress bone metastasis, we designed to harness bone’s innate anabolic response to mechanical stimulation. We studied the impact of LMHF vibration on breast cancer extravasation using our novel microfluidic co-culture platform. We showed that vibration reduced transendothelial breast cancer invasion (extravasation), suggesting that it may inhibit the early stage of bone metastasis. Furthermore, we studied the mechanism of vibration by demonstrating the importance of Piezo1 in osteocyte mechanotransduction. While the activation of Piezo1 using Yoda1 enhanced the vibration effect on cancer extravasation on Day 2, it did not further enhance it on Day 4.

Osteocytes, as the major mechanosensing cells in bones, effectively sensed LMHF vibration. We showed that vibration on osteocytes up-regulated the expression of COX-2 and down-regulated the expression of RANKL, and the overall RANKL/OPG ratio was reduced (Figure 2), consistent with the results found by Lau et al.41 In addition, we showed that vibration increased the expression of Piezo1 ion channel, which is the most highly expressed among 78 ion channels detected in MLO-Y4 osteocytes.23 Li et al. demonstrated that Piezo1 knockdown in osteocytes blunted their calcium response (we also verified in Figure S1) and diminished the up-regulation of COX-2 and OPG induced by fluid flow, but its effects under vibration remain unknown.23 In this study, we showed that Piezo1 knockdown in osteocytes significantly reduced the vibration stimulation of COX-2, OPG, and RANKL, suggesting that Piezo1 also plays a crucial role in osteocyte response to vibration (Figure 3B). However, Piezo1 knockdown did not entirely block osteocyte mechanotransduction, given that the expression of COX-2 was still modulated by vibration. This suggests that other mechanosensing mechanisms, including integrins, cilia, and other types of ion channels, may also contribute to osteocyte mechanotransduction.28 In the experiment of
siRNA-mediated Piezo1 knockdown, we observed that RANKL was up-regulated by vibration (Figure 3B), which conflicts with the results using normal osteocytes (Figure 2). It is likely that siRNA transfection reagents (lipofectamine) and conditions (serum-reduced media for 24 h) would affect RANKL response.42

Using our microfluidic platform, we were able to mimic the osteocyte-cancer microenvironment. LMHF vibration (60 Hz, 0.3 g, 1 h/day for 3 days) did not significantly affect cancer invasion if seeding cancer cells alone in the lumen channel (Figure 6A). In contrast, Yi et al. demonstrated that twice daily vibration (90 Hz, 0.3 g, 20 min) could suppress breast cancer invasion using transwell assays. The conflicting results are possible because of differences in vibration settings, duration, and assays chosen. When co-culturing cancer cells with osteocytes, vibration reduced cancer invasion via direct osteocyte regulation (Figure 6B). Interestingly, conditioned medium studies from both our group and Dwivedi et al. showed that direct signaling from flow-stimulated osteocytes would increase breast cancer migration.31,32 These seemingly contradictory observations are possibly due to differences in the experimental setup. Especially for the conditioned medium studies, the signaling is unidirectional (conditioned media containing all factors released from osteocytes were added to cancer cell cultures), whereas our microfluidic platform allows for real-time bidirectional cellular crosstalk. Second, previous studies introduced fluid flow on osteocytes alone, whereas all cells experienced vibration in this study.
Compared to the direct signaling between osteocytes and cancer cells (Figure 6B), the inclusion of endothelial cells not only improved the physiological relevance of the tissue model but also further reduced breast cancer invasion under vibration (Figure 6C). This is possible because a previous study showed that flow-stimulated osteocytes could reduce endothelial permeability, then vibration-stimulated osteocytes may achieve similar results. Further studies on endothelial cells under vibration are needed since they are in close relation with both osteocytes and cancer cells. To ensure the decrease in breast cancer extravasation was due to osteocyte regulation, we quantified cancer extravasation distances with and without osteocytes under vibration (Figure 6C). Although cancer cells can also be mechanically regulated, osteocytes are crucial for mediating vibration effects on cancer extravasation. With our experimental results, we could conclude that vibration reduced breast cancer extravasation via osteocyte regulation, suggesting that vibration may help prevent bone metastasis.

LMHF vibration demonstrated its potential in preventing breast cancer extravasation, but it may not achieve similar results in the elderly. Several studies revealed that aging provoked changes in osteocyte morphology and LCN and altered their mechanosensitivity. Piezo1 ion channels, due to their importance in osteocyte mechanotransduction (Figure 3), may serve as a target to enhance vibration effects.

Piezo1 can be chemically activated using Yoda1, a synthetic molecule discovered in 2015. Treatment of MLO-Y4 osteocytes with Yoda1 (10 μM, 2 h) increased intracellular calcium concentration and stimulated expressions of COX-2, OPG, and Wnt1, mimicking osteocyte response to fluid flow. However, in our study, the activation of Piezo1 by Yoda1 in osteocytes did not reduce cancer extravasation, indicating that Yoda1 treatment cannot fully mimic the vibration stimulation. In the combined LMHF vibration and Yoda1 therapy, Yoda1 enhanced the vibration effect on Day 2, but this was diminished on Day 4 when the vibration effect was more prominent (Figure 7). It is possible that Yoda1 accelerated the impact of vibration at the beginning and then reached a plateau. It is also possible that changes due to Yoda1
enhancement were subtle, and the fluorescent signals faded at the late time point, so we could not capture this difference in our microfluidic platform.

Piezo1 ion channels are also expressed in cancer and endothelial cells. A recent study found that Piezo1 activation in endothelial cells increased endothelial permeability in lung cancer, allowing cancer cells to easily escape through the barrier. On the other hand, Piezo1 contributes to cancer invasion and migration. For example, Piezo1 activation inhibited blebbing of MDA-MB-231 breast cancer, reducing its ameboid migration. Conversely, blockage of Piezo1 using GsMTx-4 reduced migration velocity and motility of MCF-7 breast cancer cells, suggesting that Piezo1 promoted cancer migration and invasion. Due to the unavoidable diffusion in devices in our study, Yoda1 may directly affect cancer and endothelial cell activities; hence follow-up studies are needed.

Yoda1 has yet to be FDA-approved, and its research has a long way to go before clinical studies. To demonstrate vibration efficacy and foster an earlier application, we are also interested in how vibration can be accompanied by the current standard treatment of bone metastasis (e.g., zoledronic acid (ZA), a bisphosphonate drug). Cochrane 2017 systematic review reported that the use of intravenous ZA (4 mg every 3 weeks for 1–10 years) for early breast cancer patients slightly reduced the risk of bone metastasis. Recent studies showed that ZA inhibited cancer adhesion and migration, demonstrating its antitumor effects. However, these inhibitory effects were usually achieved by using a relatively higher dose (10–100 μM of ZA in vitro, 0.5–5 μg of ZA per mouse) than recommended for humans. The high dose of bisphosphonates is often associated with severe side effects such as osteonecrosis of the jaw and, most importantly, renal toxicity. In the supplemental information, we demonstrated that in the

Figure 5. Live-dead measurement of MLO-Y4 osteocytes, HUVEC endothelial cells, and MDA-MB-231 breast cancer cells in devices under vibration
(A and B) (A) Total number of cells and (B) the proportions of live cells in total cell numbers received LMHF vibration (60 Hz, 0.3 g, 1 h/day for 3 days) or remained static. NC, Negative control of the live-dead stain. Statistical analysis: unpaired t-test in (A), one-way ANOVA in (B). n = 5. Data are represented as mean ± SD ****p < 0.0001.
presence of a relatively low level of ZA (<5 μM due to diffusion between channels), LMHF vibration could further reduce breast cancer extravasation compared with ZA alone (Figure S2). This suggests that combined vibration and ZA therapy can achieve a synergistic effect. Despite the effects of bisphosphonates on cancer progressions, they also modulated osteocyte activities by opening connexin (Cx) 43 hemichannels for ATP release. These extracellular ATPs (eATPs) suppressed breast cancer growth and progression by activating P2X receptors in cancer cells.33,56 Therefore, in addition to their direct antitumor effects, bisphosphonates also have indirect inhibitory effects on breast cancer progression via osteocyte signaling.

Most recent studies focus on osteocyte regulation of breast cancer cells. Wang et al. targeted the other way in which how osteocyte functions were affected by breast cancer cells. They observed that conditioned media from mechanical-stimulated breast cancer cells altered osteocyte mechanosensitivity by increasing dendritic formation and downstream resorption.57 However, conditioned medium studies only allow for one-way communication between cells. By using the microfluidic platform, we could better mimic bidirectional cellular crosstalk in vivo. Both this study and our previous study showed that osteocytes regulated breast cancer extravasation under mechanical stimulation, suggesting that osteocyte functions were maintained in the presence of breast cancer cells.35

Figure 6. LMHF vibration (60 Hz, 0.3 g, 1 h/day for 3 days) regulated breast cancer invasion in both direct and indirect osteocyte signaling

(A) Representative fluorescent images of MDA-MB-231 breast cancer invasion (Green, CellTracker Green) in devices. The histogram compares the cancer invasion distance over 3 days under LMHF vibration with that in static.

(B) Representative fluorescent images of MDA-MB-231 breast cancer invasion (Green, CellTracker Green) via direct MLO-Y4 osteocyte regulation in devices. The histogram compares the cancer invasion distance over 3 days under LMHF vibration with that in static.

(C) Representative fluorescent images of MDA-MB-231 breast cancer extravasation (Green, CellTrackerTM Green) through the HUVEC endothelial barrier (Red, CellTrackerTM Orange) in devices. The histogram compares the cancer extravasation distance over 3 days under LMHF vibration with that in static and no osteocyte group.

Statistical analysis: unpaired t-test in A and B, one-way ANOVA in C. n = 20+. Data are represented as mean ± SD **p < 0.01, ****p < 0.0001. See also Figure S2.
In conclusion, our microfluidic platform allows for real-time cell-cell interactions, mimicking the bone-cancer environment in vivo. This study highlights the potential of LMHF vibration utilized alone in preventing breast cancer bone metastasis or accompanied with drugs (i.e., Yoda1 or ZA) to synergistically combat breast cancer bone metastasis.

Limitations of the study
There are several limitations to this study. One limitation is the drug diffusion between channels. Yoda1 was initially added to the osteocyte channel to activate Piezo1, which is highly expressed in osteocytes. However, the drug can diffuse between osteocyte and lumen channels in the microfluidic device within 10 min; therefore, drugs would eventually affect all cell types. Although it is the situation in vivo, we were unable to isolate factors and study the underlying mechanism.

The use of both human (HUVECs and MDA-MB-231) and murine cell lines (MLO-Y4) may raise a concern about cross-species variation. However, it is common to inject MDA-MB-231 cancer cells into mice for breast cancer studies. HUVEC endothelial cells are widely used to establish an endothelial barrier for MDA-MB-231 extravasation, making our study comparable with others. Furthermore, MDA-MB-231 cells are triple-negative breast cancer (TNBC) cells that do not express estrogen (ER), progesterone (PR), and human epidermal growth factor receptor-2 (HER2). TNBCs are considered highly invasive and more aggressive than other types of breast cancers, and approximately 46% of TNBC patients have distant metastasis; hence, it is suitable for our extravasation study. However, TNBC is less common and more likely to occur in premenopausal women. Around 80% of breast cancers are ER-positive, meaning cancer growth in the presence of estrogen. Future research should include ER-positive breast cancer cells for comprehensive studies.

Lastly, although MLO-Y4 osteocytes are an established cell line for osteocyte mechanobiology, some important factors, such as SOST, are not detectable in MLO-Y4 secretions. In addition, several studies have reported an age-related decrease in osteocyte mechanosensitivity. Since the elderly (≥65 years old) represent around 50% of total breast cancer patients, studies involving aged cell lines are in dire need. Primary osteocytes isolated from young and aged mice will be used to compare vibration effects on young and aged osteocytes. We expect that the Piezo1 ion channel, due to its importance in osteocyte mechanotransduction, may serve as a target to elevate mechanosensitivity in aged osteocytes.
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105500.

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AUTHOR CONTRIBUTIONS
X.S. performed the experiments, analyzed the data, and drafted the article. C.L. coordinated the study and helped with data interpretation. X.M. designed the microfluidic co-culture platform. L.W. and L.Y. supervised the project, provided the resources, and revised the article.

DECLARATION OF INTERESTS
The authors declare no competing interests.

INCLUSION AND DIVERSITY
We support inclusive, diverse, and equitable conduct of research.

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REFERENCES
1. Lipton, A., Uzzo, R., Amato, R.J., Ellis, G.K., Hakimian, B., Roordman, G.D., and Smith, M.R. (2009). The science and practice of bone health in oncology: managing bone loss and metastasis in patients with solid tumors. J. Natl. Compr. Canc. Netw. 7, 1–29. quiz S30. https://doi.org/10.6004/jnccn.2009.0080.

2. Coughlin, T.R., Romero-Moreno, R., Mason, D.E., Nystrom, L., Boerckel, J.D., Niebur, G., and Littlepage, L.E. (2017). Bone: a fertile soil for cancer metastasis. Curr. Drug Targets 18, 1281–1295. https://doi.org/10.2174/1389450117666161226121650.

3. Shemanko, C.S., Cong, Y., and Forsyth, A. (2016). What is breast in the bone? Int. J. Mol. Sci. 17, 1764. https://doi.org/10.3390/ijms17101764.

4. Gdowski, A.S., Ranjan, A., and Vishwanatha, J.K. (2017). Current concepts in bone metastasis, contemporary therapeutic strategies and ongoing clinical trials. J. Exp. Clin. Cancer Res. 36, 108–113. https://doi.org/10.1186/s13046-017-0578-1.
5. Sheill, G., Guinan, E.M., Peat, N., and Hussey, J. (2018). Considerations for exercise prescription in patients with bone metastases: a comprehensive narrative review. PM R 10, 843–864. https://doi.org/10.1016/j.pmrj.2018.02.006.

6. Lynch, M.E., Brooks, D., Mohanan, S., Lee, M.J., Polamraju, P., Dent, K., Bonassar, L.J., Van Der Meulen, M.C.H., and Fischbach, C. (2013). In vivo tibial compression decreases osteolysis and tumor formation in a human metastatic breast cancer model. J. Bone Miner. Res. 28, 2357–2367. https://doi.org/10.1002/jbmr.1966.

7. Wang, S., Pei, S., Wasi, M., Parajuli, A., Yee, A., You, L., and Wang, L. (2021). Moderate tibial loading and treadmill running, but not overloading, protect adult murine bone from destruction by metastasized breast cancer. Bone 153, 116101. https://doi.org/10.1016/j.bone.2021.116100.

8. Mogil, R.J., Kaste, S.C., Ferry, R.J., Hudson, M.M., Mulrooney, D.A., Howell, C.R., Partin, R.E., Srivastava, D.K., Robison, L.L., and Ness, K.K. (2016). Effect of low-magnitude, high-frequency mechanical stimulation on BMD among young childhood cancer survivors a randomized clinical trial. JAMA Oncol. 2, 908–914. https://doi.org/10.1001/jamaoncol.2015.6557.

9. Marin-Cascales, E., Alcaraz, P.E., Ramos- Campo, D.J., Martinez-Rodriguez, A., Chung, L.H., and Rubio-Arias, J. (2018). Whole-body vibration training and bone health in postmenopausal women: a systematic review and meta-analysis. Medicine 97, e11918. https://doi.org/10.1097/MD.0000000000011918.

10. Oliveira, L.C., Oliveira, R.G., and Pires-Oliveira, D.A.A. (2016). Effects of body vibration on bone mineral density in postmenopausal women: a systematic review and meta-analysis. Osteoporos. Int. 27, 2913–2933. https://doi.org/10.1007/s00198-016-3618-3.

11. Rubin, C., Recker, R., Cullen, D., Ryaby, J., McCabe, J., and McLeod, K. (2004). Prevention of postmenopausal bone loss by a low-magnitude, high-frequency mechanical stimulus: a clinical trial assessing compliance, efficacy, and safety. J. Bone Miner. Res. 19, 343–351. https://doi.org/10.1359/JBMR.0301251.

12. Liang, Y.Q., Qi, M.C., Xu, J., Xu, J., Liu, H.W., Dong, W., Li, Y.J., and Hu, M. (2014). Low-magnitude high-frequency loading, by whole-body vibration, accelerates early implant osseointegration in ovariectomized rats. Mol. Med. Rep. 10, 2835–2842. https://doi.org/10.3892/mmr.2014.2597.

13. Vanleene, M., and Shelefbine, S.J. (2013). Therapeutic impact of low amplitude high frequency whole body vibrations on the osteogenesis imperfecta mouse bone. Bone 53, 507–514. https://doi.org/10.1016/j.bone.2013.01.023.

14. Rubin, C., Turner, A.S., Bain, S., Mallinckrodt, C., and McLeod, K. (2001). Low mechanical signals strengthen long bones. Nature 412, 603–604.

15. Rubin, C., Turner, A.S., Müller, R., Mittra, E., McLeod, K., Lin, W., and Qin, Y.X. (2002). Quantity and quality of trabecular bone in the femur are enhanced by a strongly anabolic, noninvasive mechanical intervention. J. Bone Miner. Res. 17, 349–357. https://doi.org/10.1359/jbmr.2002.17.2.349.

16. Shi, H.F., Cheung, W.H., Qin, L., Leung, A.H.C., and Leung, K.S. (2010). Low-magnitude high-frequency vibration treatment augments fracture healing in ovariectomy-induced osteoporotic bone. Bone 46, 1299–1305. https://doi.org/10.1016/j.bone.2009.11.028.

17. Baker, M.K., Peddle-McIntyre, C.J., Galvão, D.A., Hunt, C., Spny, N., and Newton, R.U. (2018). Whole body vibration exposure on markers of bone turnover, body composition, and physical functioning in breast cancer patients receiving aromatase inhibitor therapy: a randomized controlled trial. Integr. Cancer Ther. 17, 968–973. https://doi.org/10.1177/1534738118814499.

18. Varghese, F., and Wong, J. (2018). Breast cancer in the elderly. Surg. Clin. North Am. 98, 819–833. https://doi.org/10.1016/j.suc.2018.04.002.

19. Lynch, M.A., Brodt, M.D., and Silva, M.J. (2010). Skeletal effects of whole-body vibration in adult and aged mice. J. Orthop. Res. 28, 241–247. https://doi.org/10.1002/jor.20965.

20. Hemmatian, H., Bakker, A.D., Klein-Nulend, J., and van Lente, G.H. (2017). Aging, osteocytes, and mechanotransduction. Curr. Osteoporos. Rep. 15, 401–411. https://doi.org/10.1007/s11914-017-0402-z.

21. Schurman, C.A., Varbruggen, S.W., and Alliston, T. (2021). Disrupted osteocyte connectivity and pericellular fluid flow in bone with aging and defective TGF-β signaling. Proc. Natl. Acad. Sci. USA 118 e022999118. https://doi.org/10.1073/pnas.2029991118.

22. Yee, C.S., Schurman, C.A., White, C.R., and Hoey, D.A. (2021). Mechanically stimulated osteocytes promote the proliferation and migration of breast cancer cells via a potential CXCL1/2 mechanism. Biochem. Biophys. Res. Commun. 534, 14–20. https://doi.org/10.1016/j.bbrc.2020.12.016.

23. Zhou, J.Z., Riquelme, M.A., Gu, S., Kar, R., Gao, X., Sun, L., and Jiang, J.X. (2016). Osteocytic connexin hemichannels suppress breast cancer growth and bone metastasis. Oncogene 35, 5597–5607. https://doi.org/10.1038/onc.2016.101.

24. Reymond, N., D’Agua, B.B., and Ridley, A.J. (2013). Crossing the endothelial barrier during metastasis. Nat. Rev. Cancer 13, 858–870. https://doi.org/10.1038/nrc3528.

25. Mei, X., Middleton, K., Shim, D., Wan, Q., Xu, L., Ma, Y.H., Devadas, D., Walié, N., Wang, L., Young, E.W.K., and You, L. (2019). Microfluidic platform for studying osteocyte mechanoregulation of breast cancer bone metastasis. Integr. Biol. 11, 119–129. https://doi.org/10.1017/s175904131900008.

26. Olcum, M., and Ozcevici, E. (2014). Daily application of low magnitude mechanical stimulus inhibits the growth of MDA-MB-231 breast cancer cells in vitro. Cancer Cell Int. 14, 102–108. https://doi.org/10.1186/s12935-014-0102-z.

27. Yi, X., Wright, L.E., Pagnotti, G.M., Uzer, G., Powell, K.M., Wallace, J.M., Sarkar, U., Rubin, C.T., Mohammad, K., Guse, T.A., and Thompson, W.R. (2020). Mechanical suppression of breast cancer cell invasion and paracrine signaling to osteoclasts requires nucleo-cytoplasmic connectivity. Bone Res. 8, 40. https://doi.org/10.1038/s41413-020-00113-3.

28. Lin, C.Y., Song, X., Ye, K., Raha, A., Wu, Y., Wasi, M., Wang, L., Geng, F., and You, L. (2016). The molecular basis of bone mechanotransduction. J. Musculoskelet. Neuronal Interact. 16, 221–236.
# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological samples** | | |
| Calf serum (CS) | Gibco, USA | 16010-159 |
| EndoMax basal media | Wisent, Canada | 301-010-CL |
| EndoMax growth supplement | Wisent, Canada | 301-013-XL |
| Fetal bovine serum (FBS) | Gibco, USA | 12483-020 |
| Fibronectin | Sigma-Aldrich, USA | F1141-2MG |
| F-12K basal medium | Gibco, USA | 21127022 |
| Matrigel | Corning, USA | CACB354230 |
| Type-1 rat tail collagen | Corning, USA | CACB354236 |
| Type-1 rat tail collagen (high concentration) | Corning, USA | CACB354249 |
| 94% v/v α-MEM basal media | Wisent, Canada | 12571063 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Acetic acid | Sigma-Aldrich, USA | A6283-500mL |
| BLOCK-iT™ Alexa Fluor™ red fluorescent control | Invitrogen, USA | 14750100 |
| CellTracker™ Green | Invitrogen, USA | C2925 |
| CellTracker™ Orange | Invitrogen, USA | C2927 |
| DNase | Thermo Fisher Scientific, USA | EN0521 |
| Fura-2 AM | Abcam, UK | ab120873 |
| LightCycler 480 SYBR Green I Master Mix | Roche, Switzerland | 04707516001 |
| Lipofectamine RNAiMAX | Invitrogen, USA | 13778075 |
| Opti-MEM | Gibco, USA | 18295-062 |
| Penicillin streptomycin (P/S) | Gibco, USA | 15140122 |
| Polymethyldisiloxane (PDMS) and curing agent | Dow, USA | DC4019862 |
| SuperScript III reverse transcriptase | Invitrogen, USA | 18100-044 |
| SJ-8 2050 | Microchem, USA | Y111072 |
| SJ-8 2075 | Microchem, USA | Y111074 |
| Yoda1 | Sigma-Aldrich, USA | SML1558-5MG |
| Zeledronic acid | Sigma-Aldrich, USA | SML0223-10MG |
| 1X DPBS | Sigma-Aldrich, USA | D8537-500ML |
| 5 N NaOH | VWR International, USA | BDH7247-1 |
| 10 mM dNTP mix | Invitrogen, USA | 18427013 |
| 10X DPBS | Sigma-Aldrich, USA | D1408-500ML |
| **Critical commercial assays** | | |
| ReadyProbes™ Cell Viability Imaging Kit, Blue/Green | Invitrogen, USA | R37609 |
| RNasy Mini Kit | Qiagen, USA | 74104 |
| **Experimental models: Cell lines** | | |
| HUVEC | gift from Dr Craig Simmons, University of Toronto, Canada | N/A |
| MDA-MB-231 | ATCC, USA | HTB-26 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and request for resources, reagents, and raw data should be directed to and will be fulfilled by the lead contact, Prof. Lidan You (youlidan@mie.utoronto.ca).

Materials availability
The study did not generate new unique reagents and there are no restrictions to availability.

Data and code availability
Data and code remain available upon reasonable request to the lead contact, Prof. Lidan You (youlidan@mie.utoronto.ca).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell and culture conditions

MLO-Y4
MLO-Y4 cells, a murine osteocyte-like cell line (gift from Dr. Lynda Bonewald, Indiana University, USA), were proliferated on Petri dishes or glass slides coated with 0.15 mg/mL type-1 rat-tail collagen (Corning, USA) diluted in 0.02 N acetic acid (Sigma-Aldrich, USA). They were grown to 80% confluence in 94% v/v α-MEM basal media (Wisent, Canada) supplemented with 2.5% fetal bovine serum (FBS, Gibco, USA), 2.5% calf serum (CS, Thermo Fisher, USA), and 1% penicillin streptomycin (P/S, Gibco, USA)) before trypsinizing the cells.

HUVEC
HUVEC endothelial cells (gift from Dr. Craig Simmons, University of Toronto, Canada) were cultured in EndoMax basal media (Wisent, Canada) supplemented with 2% EndoMax growth supplement (Wisent, Canada), 10% FBS, and 1% P/S.

MDA-MB-231
MDA-MB-231 cells (ATCC, USA), a human metastatic triple-negative breast cancer cell line, were grown in F-12K basal medium (Gibco, USA) supplemented with 10% FBS and 1% P/S.
METHOD DETAILS

LMHF vibration platform

The custom-made vibration platform produced vertical and sinusoidal motion with magnitudes and frequencies of 0.1–0.3 g and 30–90 Hz, respectively (Figure 1). The electrical design was focused on selecting the piezo-electric actuator and providing the input signal to drive at the desired frequency and magnitude. The selected piezo-electric actuator (TDK Electronics AG, Germany) could achieve the maximum displacement of 230 μm, which enabled the maximal vibration magnitude of 3 g at 90 Hz. SPI protocol communication, which gave Arduino the highest data transmission rate (8 MHz), was implemented between the Arduino and the analog-to-digital converter to ensure a smooth sinusoidal output wave. The mechanical design was dictated by how the vibratory motion of a piezo actuator could be facilitated. The piezo would move the platform at a fixed displacement in a single direction to induce an acceleration. The magnitude of the acceleration could be changed by varying the displacement of the piezo by changing the voltage. LMHF vibration was set at 0.3 g and 60 Hz for one hour, which led to promising effects in reducing osteoclast formation.41 At the fixed frequency, the vibration magnitude could be monitored in real-time by a separate Arduino Uno’s onboard analog-to-digital converter.

Quantitative polymerase chain reaction (qPCR)

MLO-Y4 osteocytes were seeded at 150K cells/mL in each 35 mm plate for two days. 80% confluent osteocytes either received vibration (60 Hz, 0.3 g, 1 hour) or remained static. Cells were lysed after the vibration treatment for PCR analysis using the RNeasy Mini Kit (Qiagen, USA). After DNA digestion with DNase (Thermo Fisher Scientific, USA), they were reverse transcribed using SuperScript III reverse transcriptase (Invitrogen, USA). We performed qPCR using LightCycler 480 SYBR Green I Master Mix (Roche, Switzerland) with gene-specific primers (Table S1). Relative mRNA levels were calculated using the ΔCt method.63

Piezo1 in osteocyte mechanotransduction

Piezo1 knockdown in MLO-Y4 osteocytes

MLO-Y4 osteocytes at 70% confluence were transfected with Piezo1 siRNA (Invitrogen, USA), negative control siRNA (Invitrogen, USA), or BLOCK-iT™ Alexa Fluor™ red fluorescent control (Invitrogen, USA) using lipofectamine RNAiMAX reagent (Invitrogen, USA) diluted in Opti-MEM (Gibco, USA) as per the manufacturer’s instruction (Invitrogen, USA). Briefly, 200 nM Piezo1 siRNA or negative control siRNA was mixed with 2 mL of serum-reduced media without antibiotics (99% v/v α-MEM basal media and 1% FBS) and added to the cells seeded in a 35 mm plate for 24 hours.

Calcium response after Piezo1 knockdown

MLO-Y4 osteocytes were seeded at 350K cells/mL in each type-1 collagen coated μ-Slide for one day. At 70% confluence, cells were transfected with either Piezo1 siRNA or negative control siRNA for 24 hours as described above. On the day of calcium imaging, cells were stained with Fura-2 AM (Ex: 340 nm/380 nm, Em: 510 nm; Abcam, UK) for 40 minutes at room temperature in darkness. The stain was prepared by dissolving 50 μg of Fura-2 AM in 50 μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and diluting in 5 mL of working media (97% v/v α-MEM basal media, 1% FBS, and 1% P/S). After stain, the cells were rinsed with 1X DPBS and loaded with working media. The μ-Slide was connected to a custom-made syringe pump capable of generating oscillatory fluid flow (1.5 Pa, 1 Hz) and waited for 20 minutes to normalize the osteocyte mechanosensitivity before the experiments. Cells were imaged using EasyRatioPro (PTI, USA) during the experiment. A static baseline reading of cell response was taken for the first minute, and flow was then applied to the osteocytes for three minutes. After the experiment, the ratiometric calcium data (340 nm/380 nm) was analyzed using a previously developed MATLAB code.64 The calcium response was considered significant only if it was at least twice fold as the static baseline.

Gene expressions using qPCR after Piezo1 knockdown

MLO-Y4 osteocytes were seeded at 150K cells/mL in each 35 mm plate for two days. Cells were transfected with either Piezo1 siRNA or negative control siRNA for 24 hours as described above. Transfected osteocytes either received vibration (60 Hz, 0.3 g, 1 hour) or remained static. Cells were lysed right after the treatment for qPCR analysis as described above.
Fabrication of microfluidic co-culture platform

A microfluidic co-culture platform with a multi-layer design, previously used in our lab for another study, was re-fabricated by following standard photolithography procedures. Briefly, the platform design on a photomask was transferred onto a silicon wafer spin-coated with SU-8 2050 and 2075 (Microchem, USA) under UV exposure. The silicon wafer with imprinted designs of osteocyte, side, and lumen channels served as the mold for polydimethylsiloxane (PDMS) casting. PDMS (DOW, USA) was mixed with a curing agent (DOW, USA) at a 10:1 ratio and then cured at 60°C overnight. The PDMS device was then cut and bonded with a 75 mm × 50 mm glass slide under plasma treatment.

Osteocyte and lumen environment

The device was sterilized by rinsing with 70% ethanol and then washing with 1X DPBS. Gel coating followed the procedures published previously. Osteocyte channels were coated with a layer of 0.15 mg/mL type-1 rat-tail collagen for one hour at room temperature. All osteocyte, side, and lumen channels were then coated with 100 µg/mL fibronectin solution (Sigma-Aldrich, USA) diluted in 1X DPBS (Sigma-Aldrich, USA) for 40 minutes at 4°C. A hydrogel solution with final concentrations of 5.5 mg/mL of type-1 rat-tail collagen and 2.5 mg/mL of Matrigel was prepared by mixing 50 µL of 5X DPBS (Sigma-Aldrich, USA), 0.78 µL of 5 N NaOH (Sigma Aldrich, USA), 177 µL of 10.57 mg/mL type-1 rat-tail collagen (Corning, USA), and 102 µL of 7.6 mg/mL Matrigel (Corning, USA) on ice. The hydrogel solution was slowly loaded to the lumen channel and removed within 30 seconds. The coated device was incubated in a 37°C incubator for one hour to solidify the 3D hydrogel lumen. After gelation, all channels were filled with media and ready for cell seeding.

Live-dead measurements of cells in devices

MLO-Y4 osteocytes were seeded at 1500K cells/mL into the osteocyte channel. HUVEC endothelial cells at 2000K cells/mL or MDA-MB-231 cancer cells at 4000K cells/mL were seeded into the lumen channel. Cells in the platform received vibration (60 Hz, 0.3 g, 1 h/day for 3 days) or remained static. Negative control (NC) was prepared by treating cells with 70% ethyl alcohol for 10 minutes prior to staining. Cells were stained with 10% of NucBlue® Live (Ex: 360 nm, Em: 460 nm, Invitrogen, USA) and 10% of NucGreen® Dead (Ex: 504 nm, Em: 523 nm, Invitrogen, USA) in media and then incubated in a 37°C incubator for 30 minutes. Four random images were captured per channel using a fluorescence microscope (Nikon, Japan). The images were quantified by ImageJ.

LMHF vibration on breast cancer invasion in devices

Breast cancer invasion

MDA-MB-231 cancer cells labeled with CellTracker™ Green (Ex: 492 nm, Em: 517 nm; Invitrogen, USA) were seeded at 4000K cells/mL into the lumen channel. Cells in the platform received vibration (60 Hz, 0.3 g, 1 h/day for 3 days) or remained static. Growth media in both channels were replenished every 24 hours. The cancer invasion distances were fluorescent imaged before the treatment on Day 1 and after three-time treatments on Day 4.

Direct osteocyte regulation of breast cancer invasion

MLO-Y4 osteocytes were seeded at 1500K cells/mL into the osteocyte channel. After osteocytes were attached, fluorescent-labeled MDA-MB-231 cancer cells (CellTracker™ Green) were seeded as above into the lumen channel. Cells in the platform received vibration (60 Hz, 0.3 g, 1 h/day for 3 days) or remained static. Growth media in both channels were replenished every 24 hours. The cancer invasion distances were fluorescent imaged before the treatment on Day 1 and after three-time treatments on Day 4.

Indirect osteocyte regulation of breast cancer extravasation

HUVEC endothelial cells labeled with CellTracker™ Orange (Ex: 541 nm, Em: 565 nm; Invitrogen, USA) were seeded at 2000K cells/mL per side of the lumen channel at different orientations to coat a circular layer of the lumen channel. MLO-Y4 osteocytes and fluorescent-labeled MDA-MB-231 cancer cells (CellTracker™ Green) were seeded as above. Cells in the platform received vibration (60 Hz, 0.3 g, 1 h/day for 3 days) or remained static. Media in the lumen channels were prepared by a 1:1 mixture of HUVEC and MDA-MB-231 growth media. Growth media in both channels were replenished every 24 hours. The cancer extravasation distances were fluorescent imaged before the treatment on Day 1 and after three-time treatments on Day 4.
Combined LMHF vibration and Yoda1 therapy on breast cancer extravasation
HUVEC endothelial cells, MLO-Y4 osteocytes, and fluorescent-labeled MDA-MB-231 cancer cells (CellTrackerTM Green) were seeded in the microfluidic platform as above. Yoda1 was solubilized in DMSO and diluted in MLO-Y4 media to a final concentration of 10 μM. Osteocytes were pre-treated with either 10 μM Yoda1 or DMSO for the first one hour and then vibrated (60 Hz, 0.3 g) or remained static for another one hour in the presence of Yoda1 or DMSO per day for three days. Two hours of 10 μM Yoda1 treatment per day were used because a study showed that this treatment could activate MLO-Y4 osteocytes by increasing the expressions of mechanosensitive genes (i.e., COX-2, Wnt, and OPG).23 After the treatment, cells were rinsed with 1X DPBS and loaded with growth media (osteocyte channel: osteocyte growth media, lumen channel: 1:1 mixture of HUVEC and MDA-MB-231 growth media). The cancer extravasation distances were fluorescent imaged before the treatment on Day 1, after one-time treatment on Day 2, and after three-time treatments on Day 4.

Synergistic effects of LMHF vibration and zoledronic acid (ZA)
Live-dead measurements of osteocytes under ZA treatment
MLO-Y4 osteocytes were seeded at 15K cells/mL in each well of a 24-well plate. Cells were treated with 0, 1, 2, 3, and 5 μM ZA which was dissolved in 1X DPBS and diluted in media. Negative control (NC) was prepared by treating cells with 70% ethyl alcohol for 10 minutes prior to staining. Cells were stained with live-dead assay (Invitrogen, USA), imaged, and quantified as described above.

Effects of LMHF vibration and ZA on breast cancer extravasation
HUVEC endothelial cells, MLO-Y4 osteocytes, and fluorescent-labeled MDA-MB-231 cancer cells (CellTrackerTM Green) were seeded as above. Media in the lumen channel were prepared by diluting 10 mM ZA (Sigma-Aldrich, USA) stock in the 1:1 mixture of HUVEC and MDA-MB-231 growth media to a final concentration of 5 μM ZA. ZA with a concentration of less than 25 μM was verified previously that did not provoke MDA-MB-231 cancer cell apoptosis but reduced MDA-MB-231 cancer migration through wound healing assays.53 Osteocytes and ZA-treated cancer and endothelial cells either received vibration (60 Hz, 0.3 g, 1 h/day for 3 days) or remained static. ZA media in the lumen channel and growth media in the osteocyte channel were replenished every 24 hours. The cancer extravasation distances were fluorescent imaged before the treatment on Day 1 and after three-time treatments on Day 4.

QUANTIFICATION AND STATISTICAL ANALYSIS
In microfluidic experiments, cancer invasion or extravasation distance was quantified by measuring the change in location of MDA-MB-231 breast cancer cells (CellTrackerTM Green) before and after the treatment using ImageJ. There are five side channels per microfluidic device and six microfluidic devices per microfluidic platform. Each experiment was repeated at least twice, with at least four technical replicates in each condition. Student t-tests were used to detect statistical significance between every two conditions. For the comparison of more than two groups, ordinary one-way analysis of variance (ANOVA) or two-way ANOVA was conducted to define statistical significance. Prism nine was used for statistical analysis. Statistical significance was taken when p-value was less than 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).