

Extracellular vesicle-mediated crosstalk in bone: miR-150-5p as a mechanosensitive regulator of osteoclastogenesis

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Bone remodeling is essential for maintaining skeletal integrity by preserving the balance between bone formation and resorption, with excessive osteoclast activity contributing to osteoporosis. Osteocytes act as central regulators of osteoclastogenesis through mechanically sensitive paracrine signals, yet the influence of osteoblasts and their mesenchymal precursors remains less defined. Extracellular vesicles (EVs) have recently emerged as mediators of bone cell communication, although their role in osteoclast regulation is still underexplored. This study demonstrates that mesenchymal-derived bone cells inhibit osteoclastogenesis through an EV-dependent mechanism shaped by their differentiation stage and mechanical environment. Mechanically stimulated osteocyte-derived EVs showed the strongest anti-catabolic response. Notably, we identify miR-150-5p as a mechano-responsive miRNA enriched within osteocyte EVs, capable of inducing a dose-dependent reduction in osteoclastogenesis. Transcriptomic analyses reveal that EV treatment and miR-150-5p delivery induce substantial transcriptional changes in osteoclast precursors, including down-regulation of shared target genes linked to bone remodeling. Overall, we highlight mechanically activated osteocytes as key regulators of osteoclastogenesis through an EV-mediated mechanism, in which miR-150-5p represents a promising candidate contributor within the broader EV cargo landscape, highlighting their potential for future cell-free therapeutic strategies.

INTRODUCTION

Bone is a dynamic tissue that has the extraordinary capacity for remodeling and self-renewal to accommodate changes in its biochemical and biophysical environment, maintaining mineral homeostasis and repairing microdamage within the bone matrix. Bone remodeling arises from the coupled action of bone-forming osteoblasts and

bone resorbing osteoclasts. The balance between osteoblast-driven bone formation and osteoclast-driven bone resorption is tightly controlled, maintaining stable bone mass and mechanical strength under physiological conditions.¹ However, disruption of this balance can result in abnormal remodeling, resulting in bone pathologies such as osteoporosis and osteopetrosis.^{2,3} Osteoporosis arises from an imbalance favoring bone resorption. Driven largely by osteoclasts, this disorder leads to decreased bone mass and mineral density, deterioration of bone microarchitecture, and increased bone fragility, resulting in high fracture risk.⁴ Osteoclasts are multinucleated bone resorbing cells, and they differentiate from monocyte/macrophage precursors cells upon stimulation with macrophage colony-stimulating factor (M-CSF) and receptor activator of NF- κ B ligand (RANKL).⁵ While controlled bone resorption is essential for bone remodeling and a healthy skeleton, inhibiting osteoclast activity has been shown to be beneficial in the treatment of osteoporosis.⁶ Anti-resorptive treatments, such as bisphosphonates (BPPs), anti-RANKL antibodies (e.g., denosumab), and selective estrogen receptor modulators, function by either inducing osteoclast apoptosis or inhibiting osteoclast recruitment and activity, thereby reducing bone resorption.⁷ Although these therapies are effective in maintaining bone density, they have significant limitations, underscoring the need for alternative treatments that can effectively prevent bone loss. Osteoclast formation and function is tightly controlled by biochemical and specifically biophysical cues within the bone micro-environment, often indirectly by signals sent from other mesenchymal-derived cells within bone.⁸ Therefore, detailing how bone cells regulate osteoclastogenesis and how this may be influenced by

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their stage of lineage commitment and mechanical environment is crucial for identifying novel therapeutic strategies for bone disorders.

Bone cells, such as bone marrow stromal/stem cells (MSCs), osteoblasts, and osteocytes have been shown to play a pivotal role in regulating bone remodeling and osteoclast formation. MSCs have been found to have a dual effect on osteoclastogenesis depending on the microenvironment. *In vivo* and *in vitro* studies have demonstrated that MSCs regulation of osteoclastogenesis can occur both via direct contact and paracrine signaling.^{9,10} Under physiological conditions, MSCs can promote or suppress osteoclastogenesis via the secretion of a range of pro- and anti-osteoclastogenic cytokines.^{11,12} In addition, paracrine crosstalk between the more differentiated osteoblast and osteoclast has been proven to be essential to maintain bone homeostasis.^{13,14} Osteocytes terminally differentiate from osteoblasts and become entrapped in the newly formed bone matrix and represent the most abundant cell type in bone.¹⁵ Previous studies have identified osteocytes as master orchestrators of bone remodeling via the control of osteoblast and osteoclast activity.¹⁶ Osteocytes control osteoclastogenesis both via cell-cell interaction and signaling through the release of paracrine factors. While many studies have explored the role of mesenchymal-derived bone cells in regulating osteoclastogenesis in isolation, findings can be challenging to directly compare given the differences in cell type and environmental conditions utilized. Therefore, more targeted studies are required to systematically explore how bone cells of the mesenchymal lineage regulate osteoclastogenesis.

A potent regulator of bone remodeling is mechanical loading. Unloading or low-load environments have been shown to cause an acceleration of bone turnover, where bone-resorbing osteoclasts outpace bone-forming osteoblasts, leading to rapid bone loss. The ability of bone to sense and respond to mechanical stimuli is orchestrated by the cells of the osteogenic lineage, MSCs,¹⁷ osteoblasts,¹⁸ and particularly osteocytes.¹⁹ These cells are subjected to a constantly changing mechanical environment that comprises various biophysical stimuli, such as strain, stress, shear, pressure, and fluid flow, which they convert into biochemical signals driving cellular responses. As mentioned above, mesenchymal-derived cells regulate osteoclastogenesis mainly via paracrine signaling. Secretion of signaling molecules can change in response to a wide variety of stimuli, including mechanical stimulation.²⁰ It is widely accepted that the osteocytes are the primary mechanosensors in bone and regulate bone resorption in response to mechanical loading.^{21–23} Interestingly, recent *in vivo* studies have also demonstrated the mechanosensitive nature of both MSCs and osteoblasts, highlighting their contribution to mechanically mediated bone remodeling.¹⁸ Consistent with these findings, *in vitro* studies have demonstrated that mechanical stimulation prompts MSCs to alter their secretome, suppressing osteoclast formation, thereby contributing to rebalance bone remodeling.²⁰ Similarly, mechanical loading has been shown to modulate the osteoblast and osteocyte secretome, resulting in a mechanically driven osteoclast inhibition.^{24–27} These mechanically induced changes in the bone cell regulation of osteoclastogenesis warrant

further investigation of the specific mechanically regulated signaling components mediating these effects, as they may represent novel anti-catabolic therapies.

Extracellular vesicles (EVs) have been widely recognized as important mediators of intercellular communication.²⁸ EVs are lipid bilayer membrane-bound particles released by cells into the extracellular space both in physiologic and pathologic conditions. Over the last few decades, numerous studies have demonstrated that EVs carry and transfer a cargo of biomolecules including proteins, mRNAs, and miRNAs to regulate pathways in the target cell.²⁹ In particular, there is a growing body of evidence indicating that bone-derived EV miRNAs can interact with different signaling molecules to regulate the functions of osteoblasts and osteoclasts, thereby influencing the bone remodeling process.^{30,31} The presence of specific groups of biomolecules in EVs suggests the existence of a sorting mechanism that coordinates the selective packaging of RNAs and proteins. Biophysical cues and biochemical cues have been used to tune EV cargo and enhance their therapeutic potential.^{32,33} For instance, EVs secreted by bone cells have been shown to have a pivotal role in coordinating bone remodeling, and our group has previously demonstrated that EVs secreted by mechanically stimulated bone cells can drive an anabolic response by regulating MSCs recruitment, osteogenesis, and vessel formation. Additionally, our findings indicate that mechanical loading influences the miRNA content of osteocyte-derived EVs, pointing to a potential miRNA-associated mechanism driving regenerative effects.^{34,35} However, the role of bone cell secreted EVs and associated miRNAs in regulating osteoclastogenesis has yet to be fully explored to date.

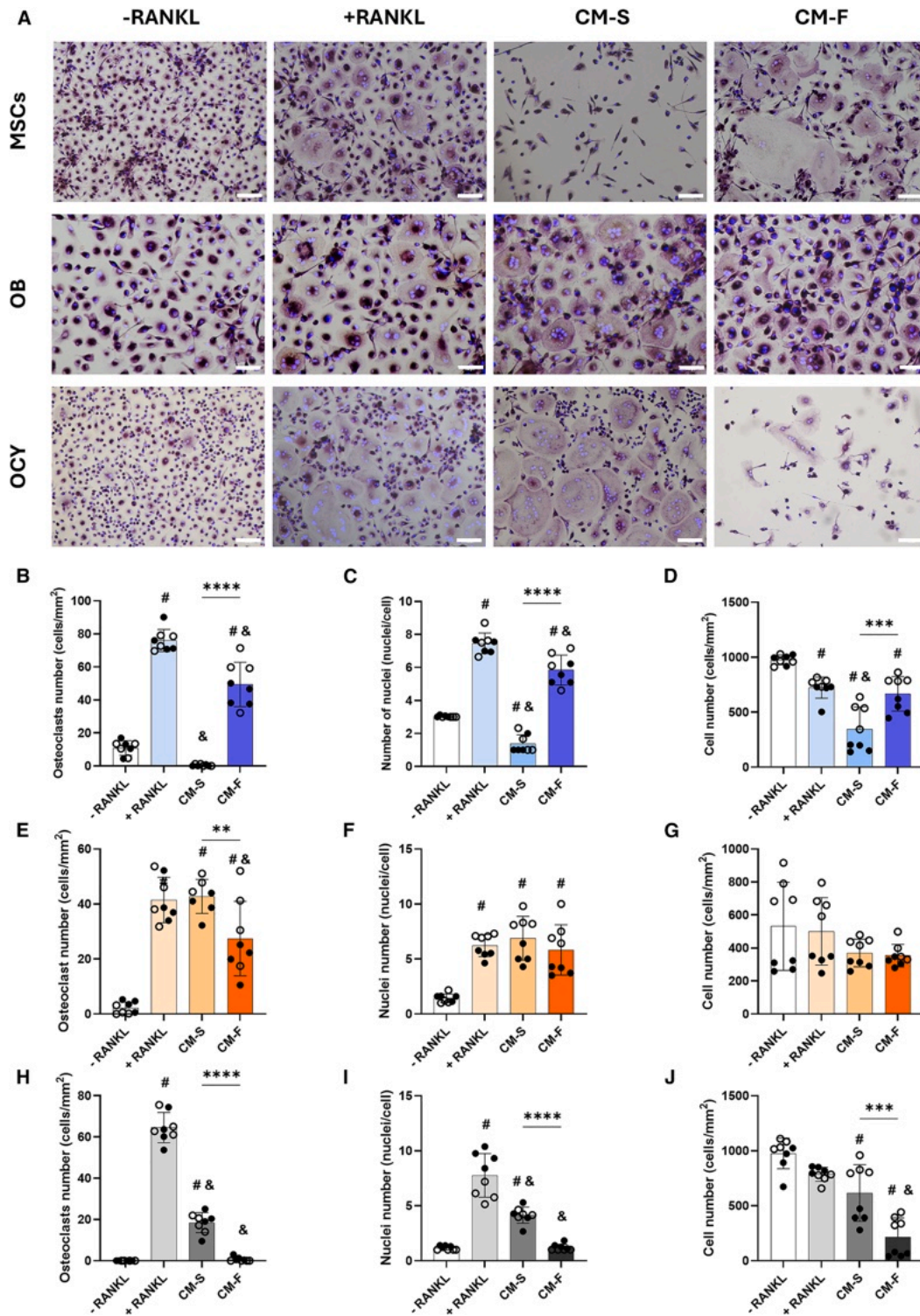
Therefore, the aim of this study is to investigate the role of mesenchymal-derived bone cells and secreted EVs in the coordination of osteoclastogenesis and to determine how the stage of lineage commitment and mechanical environment of the parent cell influences this process. Moreover, we aim to identify the signaling components within bone cell-derived EVs that may be associated with the effects on osteoclastogenesis, providing insight into potential molecular contributors and informing future therapeutic exploration.

RESULTS

Mesenchymal-derived bone cells secrete paracrine factors that regulate osteoclastogenesis in a manner that is dependent on the stage of lineage commitment and the mechanical environment

To investigate the extent to which cells of the mesenchymal lineage can regulate osteoclastogenesis, osteoclast differentiation in response to conditioned medium (CM) collected from stem/stromal cells, osteoblasts, and osteocytes cultured statically (CM-S) or exposed to 2-h oscillatory fluid shear (CM-F) was evaluated.

Human blood derived CD14⁺ monocytes cultured for 7 days in the presence of M-CSF and RANKL resulted in the formation of TRAP-positive multinucleated cells, demonstrating the ability of



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this cell type to differentiate into mature osteoclasts. Treatment with CM collected from MSCs cultured in static or mechanically stimulated conditions resulted in a significant inhibition of osteoclast formation. Specifically, the secretome from statically cultured MSCs (CM-S) demonstrated a robust near total inhibition of osteoclastogenesis (99% reduction, $p < 0.0001$) when compared with +RANKL control. Interestingly, the application of fluid shear to the MSCs (CM-F) altered the secretome, resulting in a less inhibitory response (35% reduction compared with +RANKL, $p < 0.0001$) (Figures 1A and 1B). Cell nuclei number was also assessed as a proxy for osteoclast maturity, and quantification demonstrated that osteoclast maturation was also reduced following treatment with MSCs CM when compared with +RANKL control (Figures 1A and 1C). A key step in the formation of osteoclasts is the adherence of the CD14⁺ monocyte to the surface.³⁶ It was visually evident (Figure 1A) that factors released from statically cultured MSCs reduced the adhesion of monocytes to the culture plate, which was further validated upon quantification (Figures 1A and 1D). This reduction in adhesion was not seen following treatment with CM-F. Taken together, these data indicate that statically cultured MSCs significantly inhibit osteoclastogenesis in part via the secretion of factors that reduces monocyte adhesion, an effect that is diminished following mechanical stimulation.

To assess the effect of mesenchymal lineage commitment on the regulation of osteoclastogenesis, MSCs were differentiated into osteoblasts for 14 days before CM was collected from both statically and mechanically stimulated cultures.³⁷ Unlike the parent MSCs, the secretome from statically cultured osteoblasts does not contain paracrine factors that inhibit osteoclastogenesis. However, following mechanical stimulation of the osteoblast, CM treatment results in a significant inhibition of osteoclastogenesis when compared with the static group and +RANKL control (34%, $p < 0.01$, and 36%, $p < 0.05$, reduction in osteoclastogenesis respectively) (Figures 1A and 1E). No differences in osteoclast maturity were observed between the +RANKL control and the CM-treated groups (Figures 1A and 1F). This inhibitory effect of the mechanically stimulated osteoblast secretome was not due to effects on monocyte adhesion as no difference was identified between treated groups (Figures 1A and 1G). Therefore, the commitment of MSCs to the osteogenic lineage alters the osteoclastogenic properties of the secretome.

Osteocytes represent the terminally differentiated state of the mesenchymal lineage and their role in coordinating osteoclastogenesis is well studied.³⁸ Both CM collected from osteocytes cultured in static

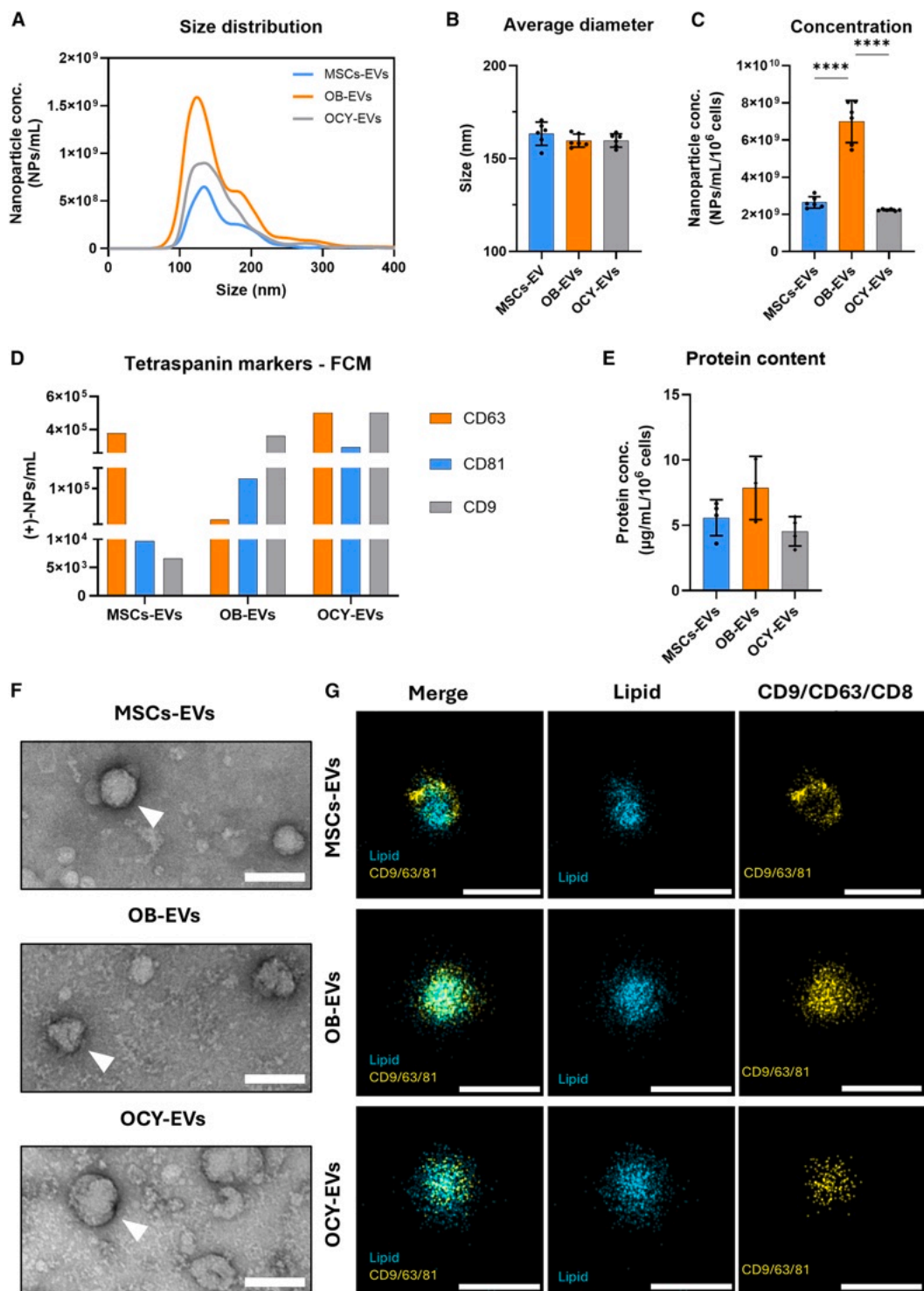
or mechanically stimulated conditions resulted in a significant inhibition of osteoclast formation. The secretome from statically cultured osteocytes elicited a 72% reduction ($p < 0.0001$) in osteoclast formation, which was further augmented following mechanical stimulation (99% reduction, $p < 0.0001$) when compared with +RANKL controls (Figures 1A and 1H). This enhanced anti-catabolic property following mechanical stimulation is consistent with that seen in osteoblasts. Similarly, nuclei number quantification identified a decreased number of nuclei upon treatment with CM-S, which was further reduced with CM-F, indicating a lower level of osteoclast maturation (Figures 1A and 1I). Interestingly, monocyte adhesion was not affected by treatment with CM collected from static osteocytes. However, treatment with CM collected following osteocyte mechanical stimulation resulted in a significant decrease in monocyte adhesion (Figures 1A and 1J). These data are consistent with the previously identified role of the osteocyte in the regulation of osteoclasts and indicate that mechanically stimulated osteocytes may inhibit osteoclastogenesis in part via the secretion of factors that reduce monocyte adhesion. Taken together, these data demonstrate that mesenchymal-derived bone cells can regulate osteoclastogenesis and maturation via a paracrine mechanism that is strongly dependent on the stage of lineage commitment and the mechanical environment at each stage of the cell lineage.

Mesenchymal-derived bone cells secrete extracellular vesicles that are of similar size but differ in quantity and surface marker expression at each stage of lineage commitment

EVs were collected and characterized from media conditioned by MSCs (MSCs-EVs), osteoblasts (OB-EVs), and osteocytes (OCY-EVs) as per MISEV guidelines.³⁹ Nanoparticle tracking analysis (NTA) analysis was performed to determine their respective size distribution and concentration. As displayed in Figures 2A and 2B, similar size distribution profiles were observed between all EV groups ranging from 100 to 200 nm with respective average diameters of 163 nm, 160 nm, and 160 nm for MSCs-EVs, OB-EVs, and OCY-EVs. Interestingly, normalizing the EV concentration to the parent cell seeding density highlighted a significantly greater EV average yield from osteoblasts (6.99×10^9 NPS/mL/ 10^6 cells) compared with EVs secreted from MSCs (2.64×10^9 NPS/mL/ 10^6 cells) or osteocytes (2.23×10^9 NPS/mL/ 10^6 cells) (Figure 2C). The morphology of these vesicles was evaluated by transmission electron microscopy, revealing nanosized particles (<200 nm) with a spherical shape (white arrow) for all three groups, which is consistent with the NTA analysis (Figure 2F). The total protein content in EV lysates was determined via a BCA assay and exhibited a similar trend in protein level to that seen in the

Figure 1. Bone cells from the mesenchymal lineage secrete paracrine factors that inhibit osteoclastogenesis in a manner that is dependent on the stage of lineage commitment and mechanical environment

(A) TRAP and DAPI staining of human monocytes following 7-day treatment with M-CSF (–RANKL); M-CSF and RANKL (+RANKL); M-CSF, RANKL, and static MSCs, osteoblast (OB), or osteocyte (OCY) conditioned media (CM-S); and M-CSF, RANKL, and mechanically activated MSCs, OB, or OCY conditioned media (CM-F). (B, E, and H) Quantification of osteoclast number (identified as TRAP+ cells with ≥ 3 nuclei), (C, F, and I) nuclei number, and (D, G, and J) total cell number for MSC, OB, and OCY CM groups, respectively. Scale bar represents 100 μ m. Values are mean $\pm$ SD for 2 different human donors ($n = 4$) (● = Donor 1, ○ = Donor 2). Statistical analysis was performed via ordinary one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, # $p < 0.01$ vs. –RANKL, & $p < 0.0001$ vs. +RANKL.



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nanoparticle concentration where there is a slight but non-significant increase in protein quantity in the OB-EVs group when compared with the MSCs and OCY-EVs (Figure 2E). Finally, the presence of tetraspanin markers (CD9, CD63, and CD81) was assessed by flow cytometry and super-resolution microscopy. All three groups were found positive for the three markers with no significant variations as the cells progress through the lineage (Figures 2D and 2G).

Mesenchymal-derived bone cells secrete extracellular vesicles that inhibit osteoclast differentiation

Given the key role that EVs play in coordinating cell-cell communication, we next sought to investigate the role that EVs may play in the above demonstrated paracrine signaling between mesenchymal-derived bone cells and osteoclasts under both static conditions (EV-S) or following mechanical stimulation (EV-F). The EV dose used was 0.5 $\mu\text{g/mL}$, equivalent to the average EV protein concentration found in the CM from each cell type. In addition to collected EVs, the effect of static and mechanically activated CM that was depleted of EVs (DM) was also investigated to assess whether soluble factors within the CM, not packaged within EVs, may influence osteoclast differentiation.

EVs released by statically cultured and mechanically stimulated MSCs significantly inhibited osteoclastogenesis, independent of the mechanical environment. Similarly, CM collected from statically cultured and mechanically stimulated MSCs that was depleted of EVs also demonstrated an inhibition of osteoclastogenesis, indicating that soluble factors not packaged within EVs can also affect this process (Figures 3A and 3B). Notably, the similar degree of inhibition observed with both EVs and EV-depleted CM indicates that osteoclastogenesis is not regulated solely by vesicle-associated cargo, but also by soluble components within the secretome. Furthermore, quantification of osteoclast nuclei showed that osteoclasts differentiated upon treatment with EVs or CM depleted from EVs (DM) displayed a decreased number of nuclei per cell (Figures 3A and 3C), which is consistent with what was identified with full CM treatment. In addition, monocyte adhesion was not found to be impaired by EVs or soluble factors contained within the DM independent of mechanical stimulation of the MSCs (Figures 3A and 3D).

Moving forward along the mesenchymal lineage, EVs secreted by osteoblasts both in static culture and following mechanical stimulation were shown to possess anti-catabolic potential. Specifically, osteoblasts cultured in static conditions produced EVs that

led to a 25% decrease in osteoclast differentiation when compared with the RANKL-treated control ($p < 0.0001$). However, mechanical stimulation of the osteoblast was shown to increase the anti-catabolic potential of the secreted EVs, resulting in a significantly more pronounced osteoclast inhibition (37% reduction, $p < 0.0001$), an effect that is consistent with that seen from the whole secretome (Figures 4A and 4B). Osteoclasts differentiated in the presence of osteoblast derived EVs showed a decreased number of nuclei when compared with the +RANKL control, indicating a lower level of maturity (Figures 4A and 4C). Furthermore, monocyte adhesion wasn't impaired upon treatment with both static and mechanically activated EVs (Figures 4A and 4D). Interestingly, treatment with EV-depleted CM (DM) led to a notable impairment in monocyte adhesion and a subsequent complete inhibition of osteoclastogenesis. This clear distinction between EV fractions and EV-depleted fractions underscores that both vesicular and soluble components contribute to the overall regulatory effect, and that EVs do not act in isolation from other secreted factors.

Interestingly, similar to that which was observed when treating human monocytes with osteoblast EVs, both static culture and mechanical stimulation of osteocytes resulted in the production of EVs possessing anti-catabolic properties. Quantification of osteoclast numbers revealed that treatment with EVs derived from statically cultured osteocytes resulted in a 54% reduction in osteoclast formation ($p < 0.0001$). EVs secreted after mechanical stimulation of osteocytes demonstrated a heightened anti-catabolic effect, resulting in a near complete inhibition of osteoclast formation (91% reduction, $p < 0.0001$) (Figures 5A and 5B). In line with what was observed with the other two EV populations, osteoclasts differentiated in the presence of osteocyte-derived EVs demonstrated a reduced number of nuclei compared with the +RANKL control (Figures 5A and 5C). Furthermore, EV treatment had no impact on the ability of monocytes to adhere. Conversely, CM depleted of EVs (DM) resulted in a substantial impairment of monocyte adhesion, subsequently leading to a lack of osteoclast formation, regardless of mechanical stimulation, in a similar manner to that identified from osteoblast-derived DM. Specifically, the reduction in monocyte adhesion was notably more pronounced when treated with mechanically activated EV-depleted CM (DM-F) (Figures 5A and 5D). These findings further emphasize that soluble, non-vesicular factors significantly influence early events such as monocyte adhesion, while EV-associated factors contribute to the modulation of osteoclast differentiation and maturation. Therefore, the comparison between EV

Figure 2. Comparative isolation and characterization of EVs derived from the conditioned media of bone marrow stromal/stem cells (MSCs), osteoblasts (OB), and osteocytes (OCY)

Nanoparticle tracking analysis (NTA) was performed to determine (A) size distribution, (B) average diameters, and (C) nanoparticle concentrations. (D) Detection of tetraspanin markers CD63, CD81, and CD9 via flow cytometry. (E) Protein content via the bicinchoninic acid assay. (F) TEM observations with scale of 100 nm (white arrow indicating EVs). (G) Super-resolution microscopy observations of a single nanoparticle with the fluorescence detection of membrane (lipid stain) and trio-tetraspanin markers (CD9/CD63/CD81) where scale bar represents 200 nm. Statistical analysis was performed via one-way ANOVA with post hoc Tukey's test, $p \leq 0.05$, $p^{**} \leq 0.005$, $p^{***} \leq 0.001$, $p^{****} \leq 0.0001$.

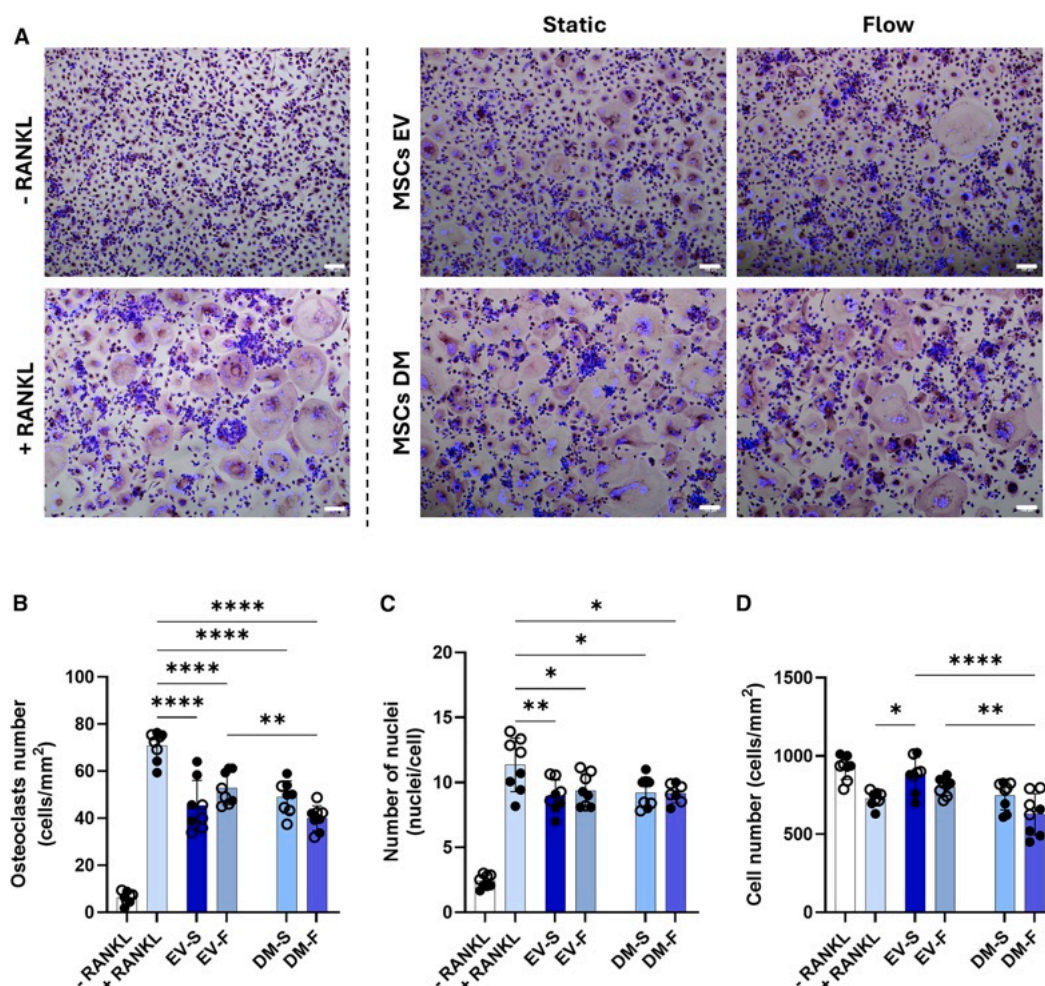


Figure 3. MSCs secrete extracellular vesicles that inhibit osteoclastogenesis independent of mechanical stimulation

(A) TRAP and DAPI staining of human monocytes following 7-day treatment with M-CSF (–RANKL); M-CSF and RANKL (+RANKL); M-CSF, RANKL, and 0.5 μ M MSCs-derived EVs (EV-S); M-CSF, RANKL, and 0.5 μ M mechanically activated MSCs-derived EVs (EV-F); M-CSF, RANKL, and static EV-depleted MSCs CM (DM-S); and M-CSF, RANKL, and mechanically activated EV-depleted MSCs CM (DM-F). (B) Quantification of osteoclast number (identified as TRAP+ cells with ≥ 3 nuclei), (C) nuclei number, and (D) total cell number. Scale bar represents 100 μ m. Values are mean $\pm$ SD for 2 different human donors ($n = 4$) (● = Donor 1, ○ = Donor 2). Statistical analysis was performed via ordinary one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

and EV-depleted fractions indicates a coordinated contribution of multiple secreted components. The inhibition of osteoclastogenesis elicited by osteocyte-derived EVs aligns with that observed upon treatment with the full osteocyte secretome, supporting an important role for EV-mediated signaling in this process, while also indicating that EVs function within the broader context of osteocyte-derived secreted factors.

Taken together, these data demonstrate that mesenchymal-derived bone cells can regulate osteoclastogenesis via an EV-mediated paracrine mechanism that is dependent on the stage of lineage commitment and mechanical environment of the EV parent cell. Importantly,

direct comparison of EV and EV-depleted fractions across all cell types highlights that both vesicular and soluble components of the secretome contribute to the observed anti-osteoclastogenic effects.

The anti-bone resorptive properties of the osteocyte-derived EVs are strongly dependent on dosage

As the osteocyte is known to be the master orchestrator of bone remodeling, the mechanically activated osteocyte-derived EVs possessed the most robust anti-catabolic response, so coupled with the fact we have previously demonstrated that these EVs possess both osteogenic and angiogenic properties,^{34,35,37} we next

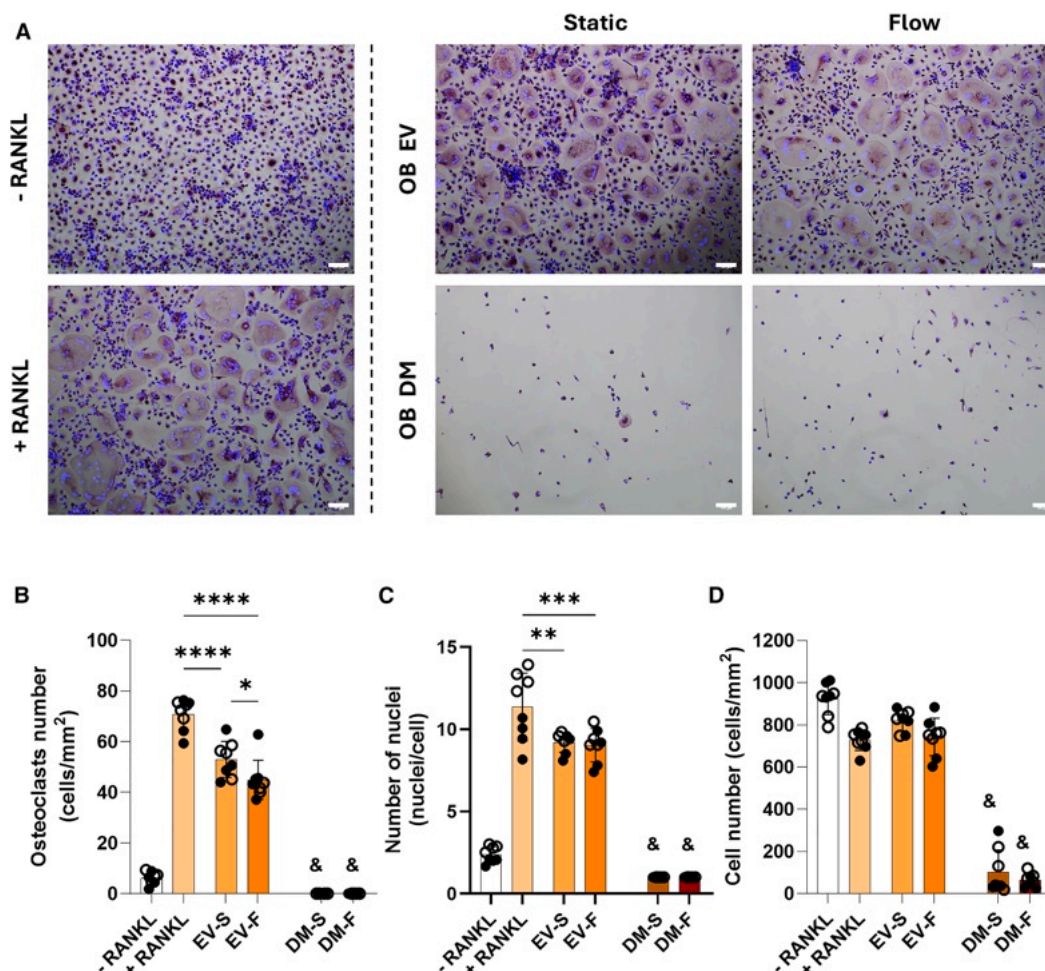


Figure 4. Osteoblasts secrete extracellular vesicles that inhibit osteoclastogenesis, where the inhibitory properties of the EVs are augmented following mechanical stimulation of the osteoblast

(A) TRAP and DAPI staining of human monocytes following 7-day treatment with M-CSF (–RANKL); M-CSF and RANKL (+RANKL); M-CSF, RANKL, and 0.5 $\mu\text{g/mL}$ static osteoblast (OB)-derived EVs (EV-S); M-CSF, RANKL, and 0.5 $\mu\text{g/mL}$ mechanically activated OB-derived EVs (EV-F); M-CSF, RANKL, and static EV-depleted OB CM (DM-S); and M-CSF, RANKL, and mechanically activated EV-depleted OB CM (DM-F). (B) Quantification of osteoclast number (identified as TRAP+ cells with ≥ 3 nuclei), (C) nuclei number, and (D) total cell number. Scale bar represents 100 μm . Values are mean $\pm$ SD for 2 different human donors ($n = 4$) (● = Donor 1, ○ = Donor 2). Statistical analysis was performed via ordinary one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, & $p < 0.0001$ vs. +RANKL, EV-S, and EV-F.

sought to explore the impact of osteocyte EV dosage on osteoclastogenesis. In this context, we treated human monocytes with increasing concentration of both static and mechanically activated osteocyte-derived EVs, namely 0.5, 1, 2, and 4 $\mu\text{g/mL}$ in the presence of both RANKL and M-CSF for 7 days. Interestingly, quantification of osteoclasts revealed that only the treatment with the lower dose of EVs resulted in osteoclast inhibition, mirroring that seen in the CM. However, inhibition was more pronounced in the presence of EVs derived from mechanically stimulated osteocytes. Conversely, doses exceeding the corresponding EVs concentration in the CM-specifically, 1, 2, and 4 $\mu\text{g/mL}$ -prompted an opposite response, enhancing osteoclast differentiation regard-

less of mechanical stimulation (Figures 6A and 6B). In addition, osteoclast nuclei number was upregulated upon treatment with all concentrations of EVs, with exception of the lowest EV dose (0.5 $\mu\text{g/mL}$), when compared with the +RANKL control group. Mechanical stimulation didn't affect osteoclast nuclei number following treatment with EV doses exceeding EV concentration in the CM, while it elicited a significant reduction following treatment with the lowest EV dose (Figures 6A and 6C). Finally, the total number of cells was not affected by any EV dose or mechanical stimulation (Figures 6A and 6D). These data suggest the presence of an optimal dose to maximize the anti-catabolic potential of osteocyte-derived EVs.

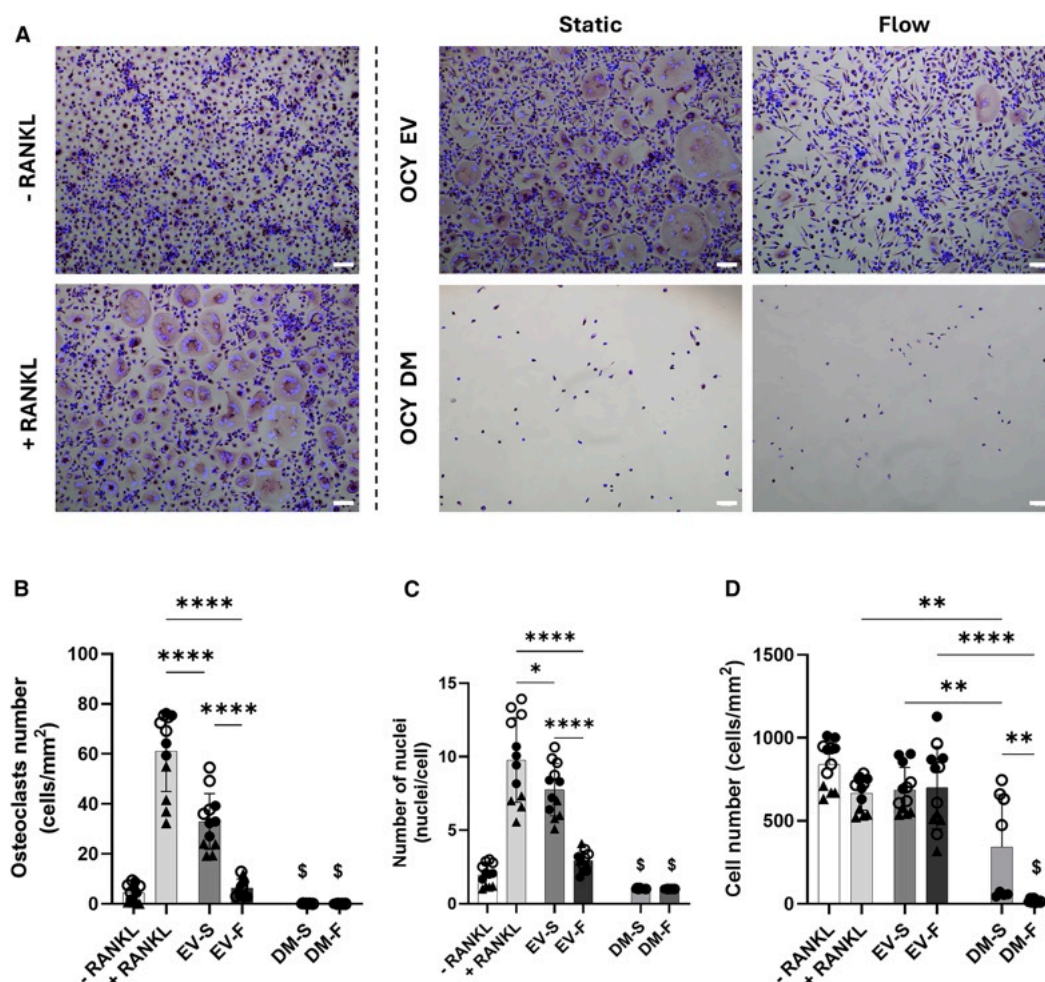


Figure 5. Osteocytes secrete extracellular vesicles that inhibit osteoclastogenesis, where the inhibitory properties of EVs are significantly augmented following mechanical stimulation of the osteocyte

(A) TRAP and DAPI staining of human monocytes following 7-day treatment with M-CSF (–RANKL); M-CSF and RANKL (+RANKL); M-CSF, RANKL, and 0.5 μ g/mL static osteocyte (OCY)-derived EVs (EV-S); M-CSF, RANKL, and 0.5 μ g/mL mechanically activated OCY-derived EVs (EV-F); M-CSF, RANKL, and static EV-depleted OCY CM (DM-S); and M-CSF, RANKL, and mechanically activated EV-depleted OCY CM (DM-F). (B) Quantification of osteoclast number (identified as TRAP+ cells with ≥ 3 nuclei), (C) nuclei number, and (D) total cell number. Scale bar represents 100 μ m. Values are mean $\pm$ SD for 3 different human donors, ($n = 4$) (● = Donor 1, ○ = Donor 2, ▲ = Donor 3). Statistical analysis was performed via ordinary one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, \$ $p < 0.0001$ vs. +RANKL and EV-S.

After evaluating the anti-osteoclastogenic properties of osteocyte-derived EVs and determining the optimal dose for maximum therapeutic efficacy, we next investigated whether EV treatment could modulate osteoclast resorptive activity. Treatment with M-CSF and RANKL (+RANKL control) resulted in resorption of 53% of the total bone slice area. However, quantification of the osteoclast-resorbed area of bone slices following EV treatment revealed that EVs derived from both static and mechanically stimulated cultures reduced osteoclast-mediated bone resorption. Notably, EVs secreted in response to osteocyte mechanical stimulation induced a significantly greater reduction in resorbed area compared with both the +RANKL control (62% reduction, $p < 0.001$) and their

static counterparts (48% reduction, $p < 0.05$). Taken together, these results showcase osteocyte-derived EVs as promising regulators of both osteoclast differentiation and bone resorptive activity (Figures 6E and 6F).

miR-150-5p is a highly expressed, mechanically regulated miRNA in osteocyte-derived EVs that possesses anti-osteoclastogenic properties

After establishing that osteocytes regulate osteoclastogenesis through an EV-mediated mechanism, and that EVs derived from mechanically stimulated osteocytes possess enhanced anti-catabolic properties, we proceeded to investigate the specific signaling

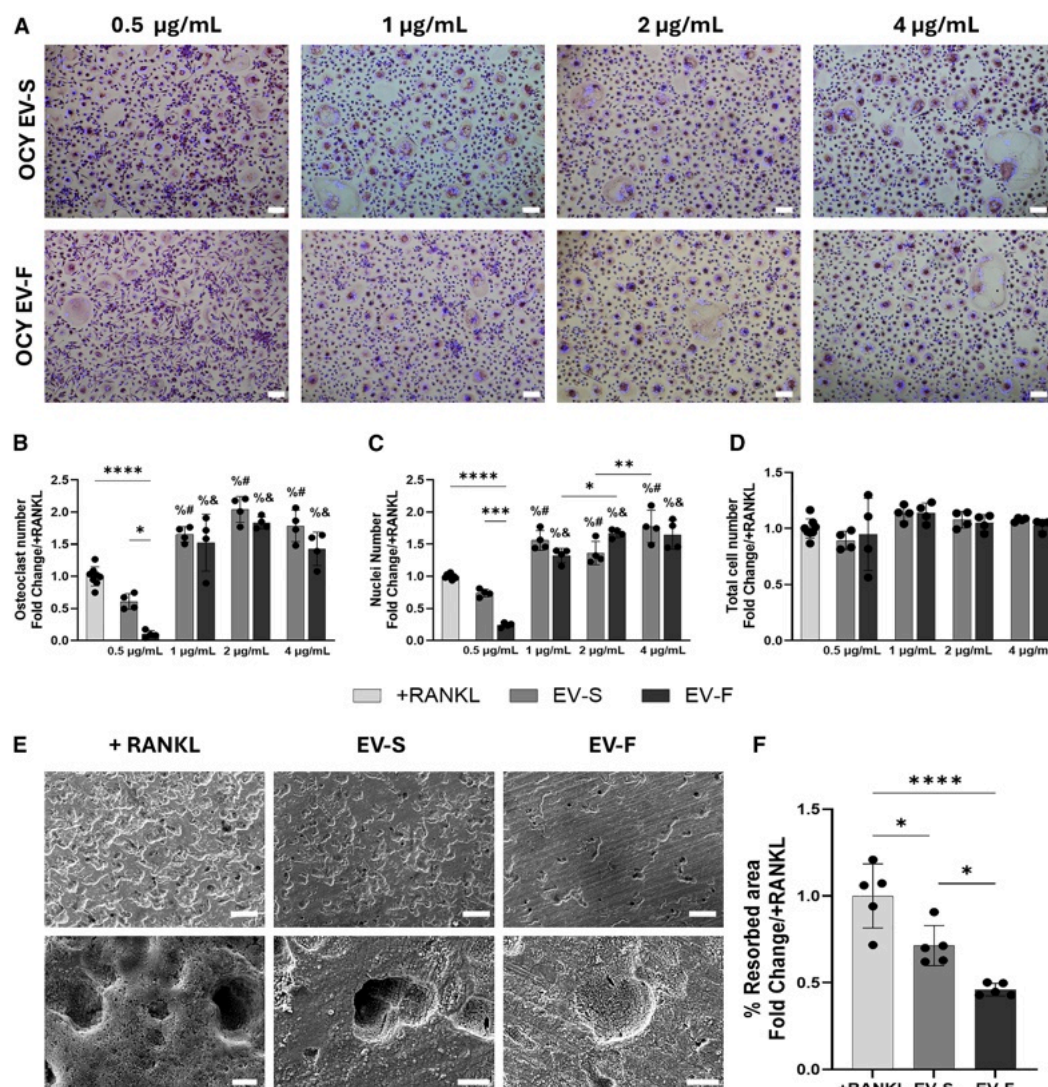


Figure 6. Optimal doses of osteocyte-derived EVs inhibit osteoclast differentiation and function

(A) TRAP and DAPI staining of human monocytes following 7-day treatment with 0.5, 1, 2, and 4 µg/mL OCY-derived EV-S and EV-F (scale bar, 100 µm). (B) Quantification of osteoclast number, (C) nuclei number, and (D) total number of cells. (E) SEM images of resorption pits on bovine bone surfaces produced by osteoclasts following OCY-EV treatment. Scale bars represent 200 µm (top row), 20 µm (bottom row), and (F) resorbed area quantification. Values are means $\pm$ SD. Statistical analysis was performed via ordinary one-way ANOVA, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, % p < 0.05 vs. +RANKL, # p < 0.001 vs. 0.5 µg/mL EV-S, & p < 0.0001 vs. 0.5 µg/mL EV-F.

components within these EVs that may contribute to the observed effects. In a previous study, we conducted miRNA sequencing on EVs collected from both static and mechanically stimulated osteocytes, identifying miR-150-5p as highly expressed and significantly upregulated in response to mechanical stimulation.³⁵ To investigate the potential role of miR-150-5p in the anti-osteoclastogenic properties presented earlier, we used TargetScan analysis and identified 356 genes that are potential targets of this mechanically regulated miRNA. Functional enrichment analysis of these genes

revealed 112 significantly enriched Gene Ontology (GO) terms. Notably, GO enrichment indicated associations with processes involved in bone regeneration, including “positive regulation of angiogenesis” (GO:0045766),⁴⁰ “canonical Wnt signaling pathway” (GO:0060070),⁴¹ and “positive regulation of nitric oxide biosynthetic process” (GO:0045429).⁴² Additionally, several GO terms were directly related to osteoclast differentiation and function, such as “osteoclast differentiation” (GO:0030316), “regulation of glycolytic process” (GO:0006110),⁴³ “cell-cell adhesion” (GO:009

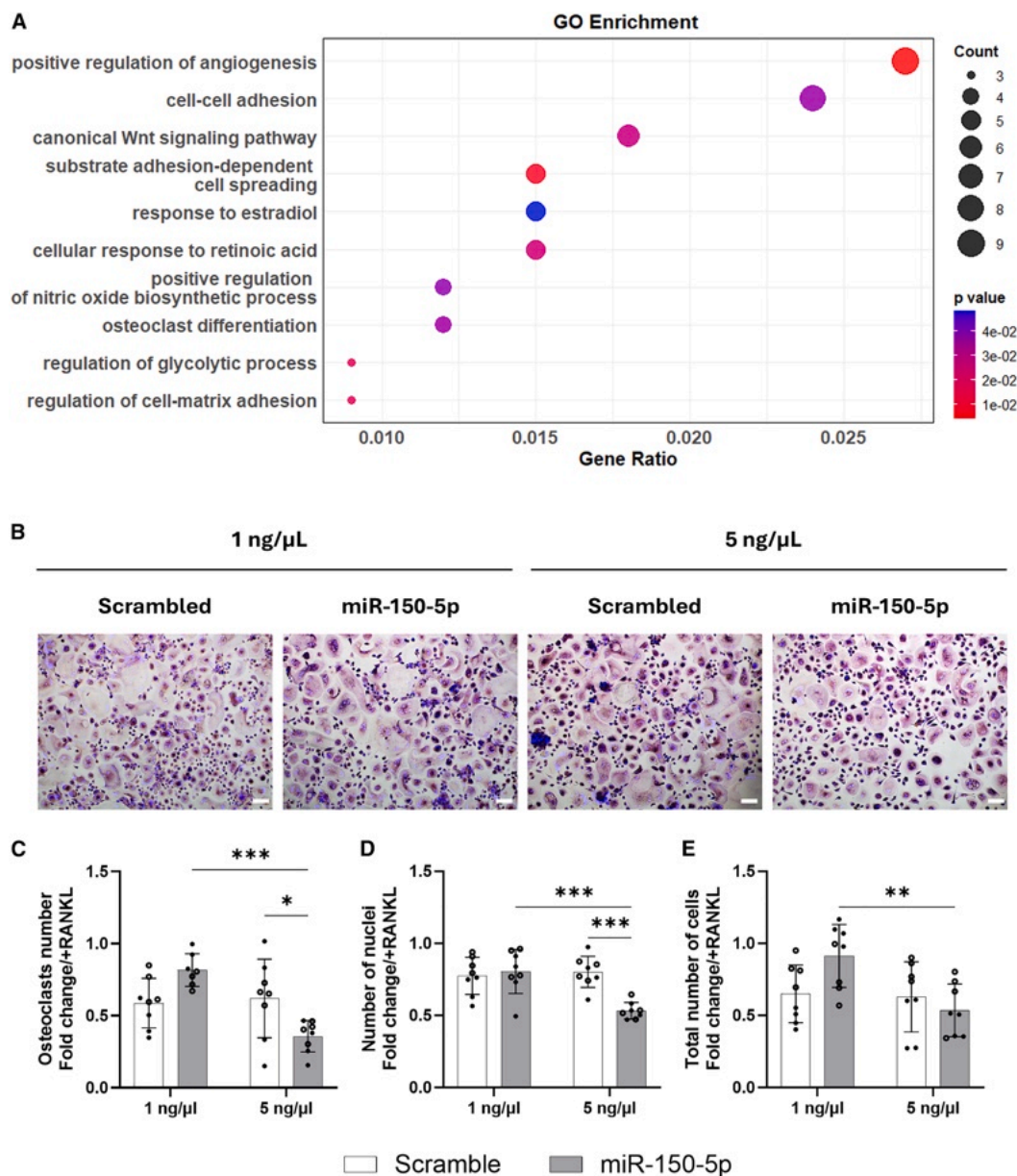
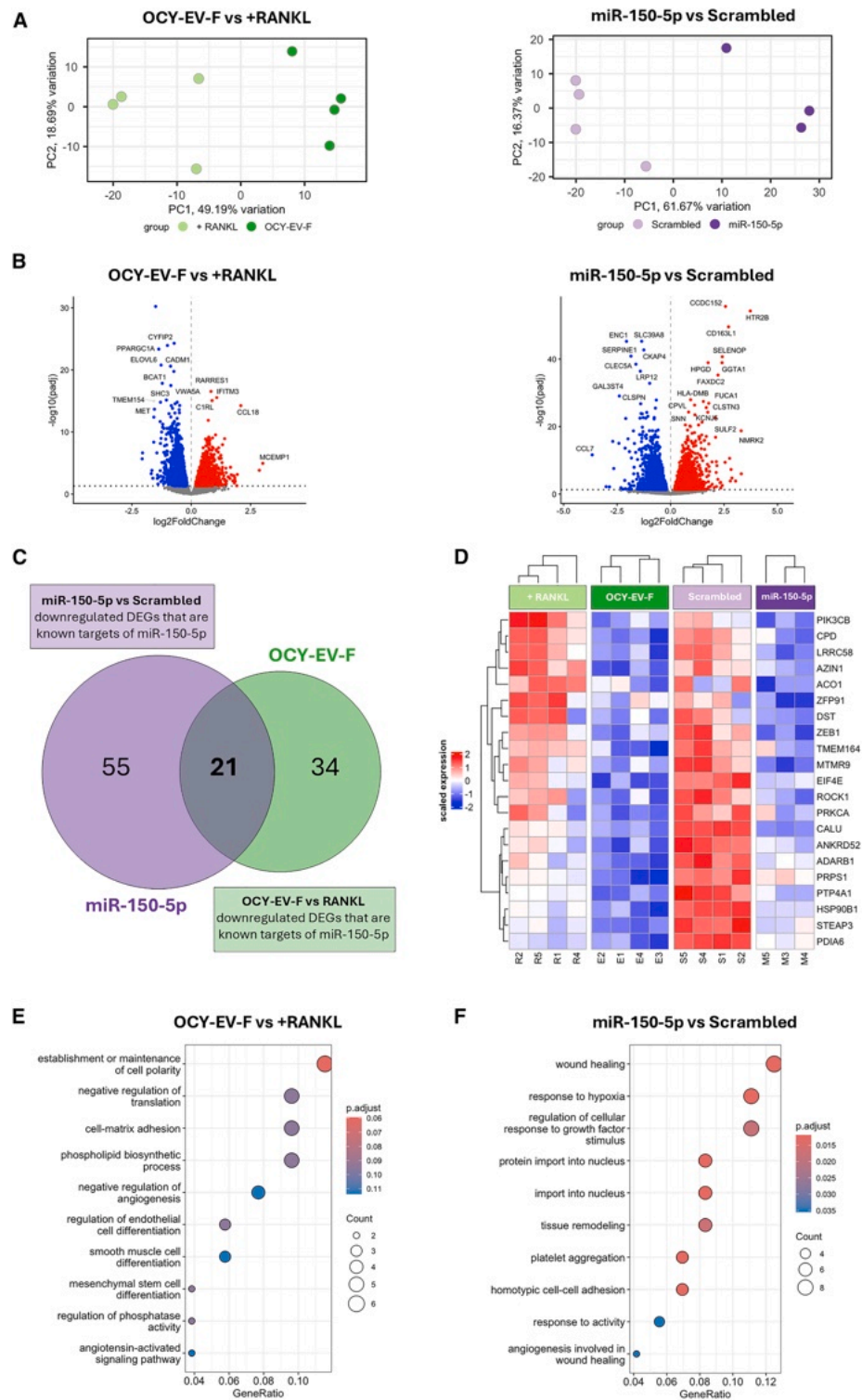


Figure 7. miR-150-5p inhibits osteoclastogenesis in a dose-dependent manner

(A) GO enrichment analysis of the target genes derived from TargetScan predictions of miR-150-5p. (B) TRAP and DAPI staining of human monocytes transfected with 1 ng/μL and 5 ng/μL scrambled miRNA control or miR-150-5p (scale bar = 100 μm). (C) Quantification of osteoclast number, (D) nuclei number, and (E) total number of cells. Values are mean ± SD for 2 different human donors ($n = 4$) (● = Donor 1, ○ = Donor 2). Statistical analysis was performed via ordinary two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

8609),⁴⁴ “regulation of cell-matrix adhesion” (GO:0001952),⁴⁵ “cellular response to retinoic acid” (GO:0071300),⁴⁶ and “response to estradiol” (GO:0032355)⁴⁷ (Figure 7A). Finally, to assess whether miR-150-5p functionally contributes to the anti-catabolic activity of the EVs, human monocytes were transfected with two different

concentrations of the miRNA (1 ng/μL and 5 ng/μL), and osteoclast differentiation was subsequently evaluated. Transfection with the lower dose of miR-150-5p (1 ng/μL) did not significantly affect osteoclast differentiation, multinucleation, or monocyte adhesion compared with the scrambled miRNA control. However,



(legend on next page)

transfection with 5 ng/ μ L of miR-150-5p significantly inhibited osteoclastogenesis, and the resulting osteoclasts displayed fewer nuclei, suggesting impaired maturation (Figures 7B–7E). These findings suggest that miR-150-5p, enriched in EVs from mechanically stimulated osteocytes, may contribute to the EV anti-catabolic effects by inhibiting osteoclast differentiation, highlighting its promise as a candidate therapeutic regulator for anti-resorptive strategies.

EV-F treatment and miR-150-5p transfection induce overlapping transcriptional programs underlying the inhibition of osteoclastogenesis

Building on the anti-osteoclastogenic effect observed following miR-150-5p transfection, we next investigated the transcriptional programs associated with osteocyte-derived EV-F treatment and miR-150-5p transfection in cells undergoing osteoclast differentiation in response to RANKL and M-CSF. Principal component analysis (PCA) revealed clear separation between the EV-F-treated samples and control cells exposed to RANKL and M-CSF alone (+RANKL control group), with PC1 accounting for 49.19% of the total variance. Similarly, PCA of samples transfected with the higher dose of miR-150-5p (5 ng/ μ L) and scrambled miRNA in the presence of RANKL and M-CSF showed distinct clustering between groups, with PC1 explaining 61.67% of the variance (Figure 8A), indicating that both treatments induce substantial transcriptional changes. Differential gene expression further confirmed these observations. Volcano plots illustrate the distribution of significantly regulated genes following osteocyte-derived EV-F treatment and miR-150-5p transfection, revealing extensive transcriptional responses under both conditions (Figure 8B). Given the complexity of these responses and the heterogeneous molecular cargo carried by EVs, we next focused on genes most likely to be directly regulated by miR-150-5p. Predicted miR-150-5p targets were obtained from TargetScan and intersected with the set of genes significantly downregulated following each treatment. Comparison of these filtered gene sets identified 21 shared targets downregulated following both EV-F treatment and miR-150-5p transfection, alongside with 34 targets uniquely downregulated by EV-F treatment and 55 targets specific to miR-150-5p transfection, indicating partial overlap in the regulatory network affected by the two treatments (Figures 8C and 8D). GO enrichment analysis of these downregulated predicted targets further revealed convergence in biological processes linked to bone and osteoclast biology. Targets downregulated following EV-F treatment (55 genes) were enriched in biological processes including cell adhesion, MSCs differentiation, phosphatase activity, and angiogenesis (Figure 8E). In parallel, targets downregulated following miR-

150-5p transfection (76 genes) were enriched in biological processes associated with cell adhesion, tissue remodeling, and angiogenesis (Figure 8F). Together, these analyses reveal convergent transcriptional and functional signatures between EV-F treatment and miR-150-5p transfection, consistent with a role for miR-150-5p in mediating part of the EV-induced regulatory response during osteoclastogenesis.

DISCUSSION

Bone is a dynamic tissue that is constantly remodeling via a tightly controlled balance between formation and resorption. This process enables bone to adapt to changing environmental or mechanical cues, adjusting bone mass to maintain an optimal strength to weight ratio. However, when bone resorption exceeds bone formation, it can lead to a net bone loss, microarchitectural deterioration, and an increased risk of fracture and/or poor fracture healing. Therefore, this study aimed to characterize how cells from the mesenchymal lineage regulate osteoclastogenesis; how this is influenced by their stage of lineage commitment; and by the dynamic mechanical cues these cells are exposed to physiologically and finally to identify the signaling components associated with this anti-catabolic potential. We demonstrated that the secretome of mesenchymal-derived bone cells inhibited osteoclast differentiation to differing degrees depending on their stage of lineage commitment and on the mechanical environment. Moreover, we have shown that the inhibition of osteoclastogenesis is mediated by EVs released by these bone cells. In addition, we identified the osteocyte as the optimal parent cell for the production of mechanically activated EVs with the most robust anti-catabolic potential and have highlighted the importance of EV dosage to efficiently regulate both osteoclast differentiation and bone resorptive activity. Finally, we demonstrated that miR-150-5p, cargo of osteocyte-derived EVs, inhibits osteoclast differentiation in a dose-dependent manner. Complementary transcriptomic profiling further revealed that both osteocyte-derived EV-F treatment and miR-150-5p transfection induce broad transcriptional responses in osteoclast precursors, with partial overlap in the repression of predicted miR-150-5p target genes associated with pathways relevant to bone remodeling. Taken together, this study substantially advances the understanding of EV-mediated regulation of osteoclastogenesis, identifying mechanically activated osteocyte-derived EVs as powerful modulators of osteoclast differentiation and offering a basis for further exploration of their potential in regulating pathological bone loss.

Bone cells secrete paracrine factors that inhibit osteoclastogenesis to differing degrees depending on their stage of lineage commitment. We demonstrated that statically cultured MSCs secrete factors that

Figure 8. Osteocyte-derived EV-F and miR-150-5p transfection induces overlapping transcriptional programs linked to osteoclastogenesis

(A) Principal component analysis (PCA) of RNA-seq data showing separation between osteocyte-derived EV-F-treated cells and +RANKL controls (left) and between miR-150-5p transfected cells and scrambled miRNA control (right). (B) Volcano plots showing differentially expressed genes following EV-F treatment (left) and miR-150-5p transfection (right). Significantly upregulated genes are shown in red and downregulated genes in blue. (C) Venn diagram showing the overlap between predicted miR-150-5p targets genes that are downregulated genes following EV-F treatment (green) and miR-150-5p transfection (purple). (D) Heatmap showing scaled expression levels of the 21 shared target genes across all samples. GO enrichment analysis showing biological processes associated with predicted miR-150-5p target genes downregulated following (E) osteocyte-derived EV-F (55 genes) and (F) miR-150-5p transfection (76 genes).

almost completely inhibit osteoclastogenesis, an effect likely mediated by a strong impairment of monocyte adhesion. Conversely, other studies provided evidence of MSCs supporting osteoclastogenesis.^{9,48} These conflicting *in vitro* findings on the impact of MSCs on osteoclastogenesis may stem from variations in the experimental approaches used. Several studies have investigated the interaction between osteoblasts and osteoclasts. In this context, we differentiated osteoblasts from the human MSCs, and our results demonstrated that statically cultured osteoblasts also did not inhibit or promote osteoclastogenesis.²⁴ These findings demonstrate a change in cell secretome following differentiation along the mesenchymal lineage from the same donor. Osteocytes differentiate from osteoblasts, and their role in the regulation of osteoclast activity is well established. Our findings demonstrate that the osteocyte secretome has a strong inhibitory effect on osteoclastogenesis. This is supported by previous work demonstrating that osteocyte-derived CM dramatically inhibited osteoclast activity, suggesting that osteocytes produce osteoclast inhibitor factors.^{49,50} Taken together, these results demonstrate that bone cells are involved in the regulation of osteoclastogenesis via paracrine signaling. In addition, the differing extents of osteoclastogenesis inhibition indicate that bone cell regulation of osteoclastogenesis is strongly dependent on the stage of lineage commitment.

Bone cells inhibit osteoclastogenesis via a mechanically driven paracrine mechanism. Osteocytes' ability to respond to mechanical stimulation and regulate osteoclast activity is well known. However, other cells of the osteogenic lineage are also known to be mechanoresponsive, indicating that they might also play a role in mechanically regulated bone remodeling, but the extent to which they can regulate osteoclastogenesis is less well understood. Recent studies demonstrated that mechanical unloading of MSCs promoted osteoclast differentiation,²⁰ while MSCs subjected to oscillatory flow-induced shear stress inhibited osteoclastogenesis.⁵¹ Similarly, our work demonstrated that MSCs subjected to fluid shear stress elicited an inhibition on osteoclastogenesis when compared with the RANKL-treated control, but to a lesser extent when compared with the static counterpart. On the other hand, Liu et al. observed that both static and dynamic pressure increased osteoclastogenesis.⁵² Differences between the studies may be attributed to differences in mechanical regimen and cell species. Osteoblasts have also been shown to be involved in the regulation of osteoclastogenesis and that mechanical cues play a key role in this process.^{18,24,53} In this context, we demonstrated that osteoblasts subjected to oscillatory fluid flow produced a secretome that inhibited osteoclast differentiation when compared with the static group, in line with previous reports employing other forms of mechanical stimulation.⁵⁴ Moreover, our results demonstrate that osteocytes subjected to oscillatory fluid flow strongly inhibit osteoclastogenesis via a mechanically driven paracrine mechanism, showing a more pronounced effect than osteoblasts in suppressing osteoclast formation.²⁶ This is supported by previous studies showing that osteocytes are mechanosensitive, and they inhibit osteoclastogenesis in response to fluid flow.^{27,55} Taken together we have demonstrated

the mechanical cues play a key role in bone cell regulation of osteoclastogenesis. When compared with that observed upon treatment with static cultured bone cell CM, these findings suggest the impact of mechanical stimulation on osteoclastogenesis may differ depending on the mesenchymal lineage stage.

Bone cells from the mesenchymal lineage can coordinate osteoclastogenesis via the release of EVs, a response that can be enhanced following mechanical stimulation. Previous studies have highlighted the pro-anabolic potential of osteoblast- and osteocyte-derived EVs and how the anabolic potential can be enhanced by mechanically stimulating the parent cell.^{34,56–58} However, a limited number of studies have investigated whether these EVs can elicit an anti-catabolic response and furthermore delineated the factors involved. Herein, we demonstrate that EVs secreted by both statically cultured and mechanically stimulated MSCs suppressed osteoclastogenesis, an effect mirrored by MSCs CM depleted of EVs. Importantly, the comparable inhibitory effects observed with EVs and EV-depleted CM highlight that soluble, non-vesicular factors also significantly contribute to osteoclast regulation. This indicates that MSCs secrete both vesicular and soluble inhibitory cues affecting early and late stages of osteoclast development. Previous studies have reported stem cell-derived EVs can suppress osteoclast differentiation and contribute to the regulation of bone resorption under various conditions, including mechanical stimulation.^{59,60} Our data add to this by showing that MSCs-derived EVs reduce osteoclast multinucleation, a hallmark of maturation, without impairing monocyte adhesion, and that their inhibitory effect is not significantly altered by mechanical stimulation. We were also able to demonstrate that by differentiating MSCs into osteoblasts, we obtained EVs with a different anti-catabolic potential. Our findings reveal that osteoblast-derived EVs inhibit osteoclastogenesis to differing degrees depending on the osteoblast mechanical environment. EVs from mechanically stimulated osteoblasts led to a more pronounced decrease in both the number and maturity of osteoclasts compared with their static counterparts.⁶¹ Interestingly, EV-depleted osteoblast-derived CM impaired monocyte adhesion and completely inhibited osteoclastogenesis. These EV versus CM-depleted comparisons reinforce that osteoclast regulation is mediated by multiple secreted components acting in concert rather than by EV cargo alone. Removal of EVs from osteoblast-derived CM may therefore disrupt the presentation or stabilization of adhesion-promoting cues necessary for monocyte attachment and spreading during the earliest stages of osteoclast differentiation. Among all cell types, osteocyte-derived EVs showed the strongest inhibitory impact on osteoclastogenesis. In particular, mechanically activated osteocyte-derived EVs inhibited osteoclast formation by 91%, aligning with prior evidence that osteocytes are the central regulators of bone resorption during mechanoadaptation.^{62,63} Importantly, comparison of EV-treated groups with their corresponding EV-depleted CM demonstrates that regulation of osteoclastogenesis cannot be attributed exclusively to vesicular cargo. While EVs exerted a strong inhibitory effect on osteoclast differentiation and maturation, EV-depleted fractions also significantly influenced early events such as monocyte adhesion, indicating that

soluble, non-vesicular factors contribute substantially to the overall anti-osteoclastogenic response. Taken together, these findings support a stage-specific working model in which components of the osteocyte secretome regulate osteoclastogenesis at distinct stages of the lineage. Several molecules identified in osteocyte secretome datasets,^{34,64} including chemokines involved in monocyte recruitment as well as adhesion-related extracellular matrix and cytoskeletal regulators, represent plausible candidates contributing to early adhesion processes. Thus, soluble secretome components and EV-associated signals appear to cooperate temporally across the monocyte-to-osteoclast lineage, with soluble factors facilitating early lineage establishment and EV cargo acting primarily as a regulator of terminal osteoclast maturation. Osteocyte-derived EVs displayed dose-sensitive behavior, with low (0.5 µg/mL) doses inhibiting osteoclastogenesis and high doses (>1 µg/mL) promoting it. This biphasic effect suggests a threshold-dependent balance between pro- and anti-catabolic factors, possibly due to changes in cargo saturation or uptake dynamics at higher concentrations.⁶⁵ Our findings indicate that optimal dosing is crucial for therapeutic use of osteocyte-derived EVs, especially in pathological states like osteoporosis where remodeling balance is disturbed. Importantly, the relative abundance and anatomical location of each osteogenic cell type must be considered when interpreting these findings in an *in vivo* context. Osteocytes represent more than 90%–95% of all bone cells and form an extensive interconnected network embedded throughout the mineralized matrix.⁶⁶ In contrast, osteoblasts are transient surface-lining cells, and MSCs are comparatively sparse progenitors residing primarily in the marrow niche. Moreover, osteocytes are uniquely positioned within the lacunar-canalicular system (LCS), where dynamic mechanical loading drives oscillatory interstitial fluid flow that directly exposes them to fluid shear stress, widely recognized as a primary physiological stimulus in bone.⁶⁷ As such, osteocytes are not only the most abundant osteogenic cell population but also the cell type most consistently and directly subjected to mechanical cues *in vivo*. Therefore, although MSCs and osteoblasts clearly contribute to the regulation of osteoclastogenesis, the integrated *in vivo* effect is likely to be strongly influenced by the dominant numerical presence of osteocytes and their heightened mechanosensitivity within the LCS. Our data support the concept that mechanical loading may preferentially amplify osteocyte-derived anti-catabolic signaling, which, in combination with inputs from MSCs and osteoblasts, ultimately determines the net osteoclastogenic outcome within bone.

To investigate molecular drivers of the EV-mediated anti-osteoclastogenic effect, we focused on miR-150-5p, which we previously found to be enriched and mechanically upregulated in osteocyte EVs.³⁵ GO enrichment analysis highlighted terms related to bone regeneration and osteoclast differentiation and function. Moreover, transfection of miR-150-5p into monocytes resulted in a dose-dependent inhibition of osteoclastogenesis and multinucleation, mirroring the effects of osteocyte EVs. Although miR-150-5p is known to be involved in osteogenesis^{68,69} and monocyte adhesion,⁷⁰ our findings suggest that miR-150-5p may also contribute to the regulation of osteoclast differentiation. Consistent with this, *in vivo* studies have shown that genetic

deletion of miR-150 leads to low bone mass and increased osteoclast numbers, supporting a physiological role for miR-150 in restraining osteoclastogenesis.⁷¹ More broadly, dysregulated miRNA expression has been reported in osteoporotic patients and experimental models, reinforcing the relevance of miRNA-mediated pathways in pathological bone remodeling.⁷² In addition, transcriptomic analysis revealed that both osteocyte-derived EV-F treatment and miR-150-5p transfection were associated with substantial transcriptional changes in osteoclast precursors. Given the extensive number of genes affected under each condition and the heterogeneous molecular cargo carried by EVs, we next sought to identify genes most likely to be directly regulated by miR-150-5p. Because miRNAs predominantly exert their regulatory effects through repression of target mRNAs,⁷³ we focused our analysis on genes significantly downregulated following treatment. These genes were intersected with predicted miR-150-5p targets obtained from TargetScan in order to enrich for candidate genes that may represent direct mediators of miR-150-5p activity. Among the shared targets identified through this approach, several genes have previously been implicated in osteoclast biology and bone remodeling. For example, the transcription factor *ZEB1* has been shown to regulate osteoclast activation and metabolic control of bone resorption.⁷⁴ Similarly, *PIK3CB* encodes the PI3Kβ catalytic subunit, which plays an essential role in osteoclast survival differentiation and bone resorptive function *in vivo*.^{75–77} Cytoskeletal remodeling pathways also emerged among the shared targets, including *ROCK1*, a key component of the RhoA/ROCK signaling pathway that regulates osteoclast cytoskeletal organization and sealing zone formation required for bone resorption.^{78–80} Additional shared targets further support the relevance of this gene set to osteoclast biology. Members of the HSP90 family, including *HSP90B1*, have been shown to promote osteoclastogenesis through stabilization of signaling complexes and activation of NF-κB pathways.⁸¹ *PRKCA* (protein kinase C-α) is also known to regulate osteoclast adhesion, migration, and bone resorptive activity through PKC-dependent signaling and cytoskeletal remodeling.⁸² Finally, *DST* (dystonin) encoding for BPAG1, a cytoskeletal linker protein, has recently been reported to promote osteoclastogenesis and contribute to postmenopausal bone loss through ERK signaling pathways.⁸³ Taken together, the presence of these genes among the shared downregulated targets strengthens the biological plausibility that miR-150-5p may contribute to the EV-mediated anti-osteoclastogenic effect by modulating transcriptional networks involved in osteoclast adhesion, cytoskeletal organization, signaling, and resorptive activation. While other targets identified in the analysis may also participate in these regulatory processes, their direct role in osteoclast biology remains less well characterized and warrants further investigation. Collectively, this study expands miR-150-5p functional repertoire and therapeutic potential and suggests that osteocyte EV-mediated inhibition may be at least partially linked to cargo-specific miRNAs.

One potential limitation of this study is the use of a murine osteocyte cell line to generate osteocyte-derived EVs. However, our data demonstrate similar trends in osteoclast formation inhibition and monocyte adhesion following treatments with human osteoblast- and murine

osteocyte-derived EVs and DM, which differs from the effects observed with human MSCs-derived EVs and DM. This suggests that the MLO-Y4 cell line may serve as a reliable model for human osteocytes. In addition, from a therapeutic prospective, previous research has explored the use of plant-derived EVs in human cell treatments, reporting no adverse effects, while supporting human cell proliferation, reducing inflammation, and apoptosis.⁸⁴ Additionally, reported findings have demonstrated that EVs from human cells can be functionally active and well tolerated in rodent models,⁸⁵ suggesting that EVs from different species may represent a safe and viable therapeutic approach for human applications.

In conclusion, this study shows that MSCs, osteoblasts, and osteocytes secrete EVs with the potential to inhibit osteoclastogenesis, although the extent and mechanism of action vary by cell type and mechanical environment. Osteocytes, consistent with their role of master orchestrators of bone remodeling, secrete the most potent EVs, regulating both osteoclast differentiation and function, particularly in response to mechanical cues, a response that is potentially mediated via the mechanical regulation and EV packaging of miR-150-5p. Combined with the demonstrated anabolic and angiogenic properties of these EVs, mechanically activated osteocyte-derived EVs represent a novel multitargeted therapeutic for bone regeneration.

MATERIALS AND METHODS

Cell culture

Mesenchymal stromal/stem cell culture

Human bone marrow stromal/stem cells (MSCs) were isolated from bone marrow (Lonza) and cultured in Dulbecco's modified eagle medium (DMEM) with GlutaMAX (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (P-S) (Sigma). Cells were cultured at 37°C and 5% CO₂ and used between passage 3–5 were for all the experiments, as previously described.^{34,86}

Osteoblast culture and differentiation

MSCs were seeded at 6,500 cells/cm² in a 6-well plate (Sarstedt) and cultured in DMEM GlutaMAX (Gibco) supplemented with 10% FBS and 1% P-S at 37°C and 5% CO₂. 100 nM dexamethasone, 0.05 nM L-ascorbic acid, and 10 mM β-glycerol phosphate were added to the medium to induce osteogenic differentiation and were cultured for a further 14 days to obtain osteoblasts. At the endpoint, osteoblasts were washed in PBS, and then 1 mL/well of 2 mg/mL collagenase type II in PBS (Gibco) was added for 30 min to disrupt the deposited matrix. Collagenase was then removed and 1 mL/well of trypsin (Sigma) was added to lift the osteoblasts for reseeding onto glass slides prior to mechanical stimulation.

Osteocyte culture

As human osteocytes are challenging to obtain in sufficient number and purity, the murine osteocyte-like MLO-Y4 cell line (Kerafast) was chosen for this study.⁸⁷ MLO-Y4 cells maintained in MEM Alpha (α-MEM) (Merk) supplemented with 2.5% FBS (Gibco),

2.5% Calf Serum (CS) (Biosera), 1% P-S (Sigma), and 1% L-glutamine (L-G) (Sigma) as previously described.⁸⁸ Cells were cultured at 37°C and 5% CO₂ and used for experiments when they had reached 80% confluency between passages 40–60.

Mechanical stimulation and conditioned media collection

Glass slides (75 × 38 mm) (Corning) were sterilized in 70% IMS for 1 h, allowed to dry, and coated with rat tail collagen type I (0.15 mg/mL) (Corning) for 1 h. Prior to seeding a wash step was performed with PBS (Sigma). MLO-Y4 cells were seeded with a density of 11,600 cells/cm² onto the coated glass slides. MSCs and osteoblasts were seeded independently on the coated glass slides at a density of 6,140 cells/cm². After 24-h culture, cells were treated with serum-reduced media overnight. Osteocyte media consisted of α-MEM supplemented with 0.5% FBS, 0.5% CS, 1% L-G, 1% P-S, while the MSCs and osteoblast media consisted of DMEM GlutaMAX supplemented with 0.5% FBS and 1% P-S. The slides were then placed in a custom-made parallel plate flow chamber (PPFC) bioreactor as previously described.⁸⁹ Briefly, the PPFCs were connected to a syringe pump to mechanically stimulate the cells by applying an oscillatory fluid flow that included shear stress of 1 Pa at 1 Hz for 2 h. Cells were also cultured statically in the PPFCs for 2 h as a control. Following stimulation, the chambers were disassembled, and the slides were washed with PBS and put in custom-made slide chambers with 2.5 mL of α-MEM supplemented with 0.5% EV-depleted FBS (dFBS), 0.5% EV-depleted CS (dCS), 1% L-G, and 1% P-S for osteocytes and DMEM supplemented with 0.5% dFBS and 1% P-S for MSCs and OBs. After 24 h of culture, static conditioned media (CM-S) and fluid shear stimulated conditioned media (CM-F) were collected and centrifuged at 3,000 × g for 10 min at 4°C to remove debris. Supernatant was collected and stored at –80°C.

Bone cell regulation of osteoclastogenesis

The use of human blood samples for this study was approved by the research ethics committee of Trinity College Dublin and was conducted in accordance with the Declaration of Helsinki. Leukocyte-enriched buffy coats from anonymous healthy donors were obtained with permission from the Irish Blood Transfusion Service (IBTS), St. James's Hospital, Dublin (Researcher code: 0000RES625). Donors provided informed written consent to the IBTS for their blood to be used for research purposes. Peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte-enriched human buffy coats using density gradient centrifugation with LymphoPrep™ solution (Progen). CD14⁺ monocytes were isolated from PBMCs by magnetic sorting using MagniSort Human CD14 Positive Selection Kit (Invitrogen) according to the manufacturer's instructions. CD14⁺ monocytes were seeded at 625,000 cells/cm² in 96-well plates in α-MEM containing 10% FBS, 1% P-S, and 1% L-G supplemented with 25 ng/mL M-CSF (Myltenyi Biotec) and 50 ng/mL Recombinant Human sRANK Ligand (RANKL) (PeproTech) (+RANKL) induced osteoclastogenesis for 7 days. Groups cultured in growth media supplemented with 25 ng/mL M-CSF acted as a negative control for osteoclastogenesis (–RANKL). To assess the effect of bone cell secretome on osteoclastogenesis, CM collected from statically or

mechanically stimulated cells was supplemented with 9% FBS and added in a ratio of 1:1 along with growth media in presence of 50 ng/mL RANKL and 25 ng/mL M-CSF. Media and CM were replenished every 3.5 days. At day 7, cells were fixed with 2% paraformaldehyde (PFA) for 15 min at room temperature and then washed twice with PBS and stained for tartrate-resistant acid phosphatase (TRAP) activity with a leukocyte acid phosphatase (TRAP) Kit (Sigma) according to the manufacturer's protocols. Finally, cells were incubated with Triton X-100 0.1% (Sigma) for 10 min, rinsed with PBS, and then incubated with DAPI (1:2,000) (Sigma) for 10 min. TRAP-positive multinuclear cells containing 3 or more nuclei were counted as osteoclasts. Cells were imaged using an Olympus IX83 inverted microscope with 10 $\times$ objective, and osteoclasts, number of nuclei per osteoclast, and total number of cells were manually counted using the cell counter plugin on ImageJ software (version 1.54p, NIH, USA).

Extracellular vesicle collection

EVs were collected from CM via ultracentrifugation using a 70Ti-fixed angle rotor as previously described.³⁵ Briefly, CM was centrifuged at 2,000 $\times$ g for 15 min at 4°C and then filtered through a 0.45- μ m pore filter. Next, CM was ultracentrifuged at 110,000 $\times$ g for 75 min at 4°C. The resulting EV pellets were washed in PBS and ultracentrifuged again at 110,000 $\times$ g for 75 min at 4°C. Finally, EV pellets were resuspended in PBS and stored at -80°C.

Extracellular vesicle characterization

Bicinchoninic acid assay

The EV sample of 50 μ L was suspended in 50 μ L of cell lysis buffer (Invitrogen) and 25 $\times$ proteinase inhibitor (Roche). EV lysate was incubated on ice for 30 min, vortexing every 10 min for 10 s. After 30 min, the sample was centrifuged at 13,200 rpm at 4°C for 10 min. The supernatant was transferred to a new tube and stored at -20°C until required. EV lysate was quantified using the Bio-Rad protein assay dye reagent (Bio-Rad). Bovine serum albumin standards of 12.5–800 μ g/mL were prepared in PBS and used to calculate the protein content of the samples. Lysed EVs were diluted 1:2 in PBS, and whole-cell lysates were diluted 1:20 in PBS. 10 μ L of standards and sample was added to a 96-well plate in duplicate. Bio-Rad dye reagent was diluted 1:5 in deionized water, and 200 μ L was added to each standard and sample. Absorbance was read at 570 nm using a fluoStar optima microplate reader (Serial #: 08-100-241), and protein levels were calculated from the standard curve.

Flow cytometry

EV surface antigens were exposed to antibodies diluted in 0.22- μ m-filtered PBS with 2% dFBS supplemented with protease inhibitor and phosphatase inhibitor (IFCM buffer). The antibodies used were anti-CD63 conjugated with FITC (1:150) (Biolegend), CD9-PE (1:5,000) (Biolegend), and CD81-PE-Cy7 (1:150) (Biolegend). The EVs were incubated with the antibodies for 45 min at room temperature in the dark and washed using a 300-kDa filter (Nanosep), resuspended in 50 μ L IFCM buffer, and acquired within 2 h on the ImageStream X MK II imaging flow cytometer (Amnis/Luminex, Seattle, USA)

at 60 $\times$ magnification and low flow rate. EV-free IFCM buffer, unstained EVs, single-stained controls, and fluorescence minus one controls were run in parallel. Fluorescence was within detection linear range in the following channels: FITC was measured in channel 2 (B/YG_480–560 nm), PE in channel 3 (B/YG_560–595 nm), PE-Cy7 in channel 6 (B/YG_745–780 nm), and APC in channel 11 (R/V_642–745 nm). Bright field was in channel 1 and 9 (B/YG_435–480 and R/V_560–595 nm filter, respectively) and side scatter channel in channel 12 (R/V_745–780 nm 49 filter). Data analysis was performed using IDEAS software v6.2 (Amnis/Luminex, Seattle, USA). EVs were gated as SCC-low vs. fluorescence and then as non-detectable bright field (fluorescence vs. raw max pixel bright field channel), and gated EVs were confirmed in IDEAS Image Gallery.

Nanoparticle tracking analysis

Nanoparticle tracking analysis was performed on EV suspensions using the NTA NS500 system (NanoSight, Amesbury, UK) to determine particle size based on Brownian motion. EVs samples were diluted at 1:50 in PBS and injected into the NTA system, which obtained four 40-s videos of the particles in motion. Videos were then analyzed with the NTA software to determine particle size.

Transmission electron microscopy

A 20- μ L sample of EV suspension was placed onto parafilm (Sigma-Aldrich). A formvar carbon-coated nickel grid (Ted Pella) was placed on top (coated side facing the droplet) of the EV suspension droplet. The grid was incubated for 60 min at room temperature and washed in 30 μ L of PBS (3 times) on parafilm for 5 min. Absorbent paper was used to remove excess PBS from the washing steps. A droplet of PFA (2%) was placed on parafilm, and the grid was placed on top and fixed for 10 min. The PBS washing steps were repeated. The grid was then contrasted in 2% uranyl acetate (BDH), and all images were taken using the JEOL JEM-2100 transmission electron microscope at 120 kV.

Super-resolution dSTORM microscopy

Super-resolution fluorescent microscopy analyses of EVs were performed with a 100 $\times$ oil immersion 1.45 NA objective using a Nanoimager S Mark II microscope from ONI (Oxford Nanoimaging, Oxford, UK) equipped with the following lasers: 405 nm/150 mW, 488 nm/1 W, 560 nm/500 mW, and 640 nm/1 W. Fluorescence excited by 488, 560 and 640 lasers was recorded using band pass filters 498–551 nm, 576–620, and 665–705 nm respectively. Fluorescence excited by 488, 560, and 640 lasers was recorded using band pass filters 498–551nm, 576–620, and 665-705 nm, respectively. ONI EV Profiler Kit 2 (EVP2) was used to prepare EV samples for dSTORM per manufacturer's instruction. This kit includes panEV stain (membrane dye), tetraspanin antibodies (CD9, CD63, CD81), capture chips, capture reagents, and the ONI BCubed dSTORM Imaging Buffer used in this study. Three-channel (647, 560, and 488) dSTORM data (1,000 frames per channel) were acquired sequentially using AutoEV CODI acquisition software using 180-mW power at the objective for 488-nm laser, 45 mW for 560-nm laser, and 170 mW for 640-nm laser at 40-ms exposure per frame. For panEV stain,

3,000 images were acquired. Before each imaging session, bead slide calibration was performed to align fluorescent channels, achieving a channel mapping precision smaller than 10 nm. Single-molecule localization, clustering, and positivity counting were performed using the default EVP2 app setting within the CODI software provided by ONI (<https://alto.codi.bio>). Data were exported as a report and csv file for further analysis.

Extracellular vesicle regulation of osteoclastogenesis

To determine whether EVs released by mesenchymal-derived bone cells influence osteoclastogenesis, human-blood-derived CD14⁺ monocytes were seeded at 625,000 cells/cm² in 96-well plates in α -MEM containing 10% dFBS, 1% P-S, and 1% L-G (growth media) supplemented either with 25 ng/mL M-CSF (Myltenyi Biotec) and 50 ng/mL RANKL (PeproTech) (+RANKL) to induce osteoclastogenesis for 7 days or with 25 ng/mL M-CSF alone (–RANKL) as a control. EV-enriched medium was prepared by supplementing growth media with 0.5 μ g/mL of MSCs, osteoblast- and osteocyte-derived static EVs (EV-S), and fluid shear stimulated EVs (EV-F) in the presence of M-CSF and RANKL. To assess the effect of secreted soluble factors not packaged within EVs, CM-S and CM-F depleted of EVs (DM-S and DM-F) were supplemented with dFBS and added in a ratio of 1:1 along with growth media in presence of both M-CSF and RANKL. Media and EVs were replenished after 3.5 days. Osteoclast differentiation was assessed by TRAP and DAPI staining as described above. Cells were imaged using Olympus IX83 inverted microscope with 10 $\times$ objective, and osteoclasts, number of nuclei per osteoclast, and total number of cells were manually counted using the cell counter plugin on ImageJ software (version 1.54p, NIH, USA).

To assess the influence of EV concentration on osteoclastogenesis, EV-enriched medium was prepared by adding 0.5, 1, 2, and 4 μ g/mL of both static and fluid shear-stimulated osteocyte-derived EVs to the growth media. Human-blood-derived CD14⁺ was cultured in EV-enriched media at a ratio of 1:1 along with growth media in the presence of M-CSF and RANKL for 7 days as described above. Osteoclast were stained for TRAP and DAPI staining and imaged using an Olympus IX83 inverted microscope with 10 $\times$ objective, and quantification was performed using ImageJ software (version 1.54p, NIH, USA).

Osteoclast resorption activity assay

Osteoclast activity was assessed by determining the area of resorption on bovine bone slices. Briefly, bone slices (6 mm diameter, 200–220 μ m thickness) were cut from bovine cortical femur with a low-speed water-cooled diamond saw (Beuhler). 24 h before seeding, bone slices were sterilized in 70% ethanol, dried, and incubated in medium overnight. Subsequently, CD14⁺ monocytes were cultured on the sterile bone slices in α MEM supplemented with 10% dFBS, 1% P-S, and 1% L-G in the presence of M-CSF (25 ng/mL) and RANKL (50 ng/mL) for 14 days. The effect of osteocyte-derived EVs on osteoclast resorption activity was assessed by adding EV-enriched media, containing 0.5 μ g/mL EV-S and EV-F in a ratio of 1:1 with growth

media, in the presence of M-CSF and RANKL. Media and EVs were replenished every 3.5 days. After culture, samples were washed with PBS, and 200 μ L of deionized water was added for 30 min. Subsequently, the surface of the samples was scraped with a cotton swab to remove cells. Finally, samples were serially dehydrated in an increasing concentration of ethanol and a final drying step in hexamethyldisilazane to remove all water before being imaged by scanning electron microscopy (SEM). After dehydration, samples were left to dry on adhesive double-sided carbon tape attached to 11.7-mm-diameter aluminum stubs (Micro to Nano) overnight. Samples were then coated with a 5-nm-thick coating of gold/palladium using a sputter coater and imaged in a Zeiss Ultra scanning electron microscope (Zeiss). Representative images were taken using a secondary electron detector with an accelerating voltage of 10 kV. Images for quantitative analysis were taken using an in-lens detector under identical conditions. Resorption pit area was calculated using ImageJ software (version 1.54p, NIH, USA).

Bioinformatic analysis

Building on our previous findings highlighting that mechanical stimulation of osteocytes led to the productions of EVs significantly more enriched in miR-150-5p compared with those from static condition,³⁵ miR-150-5p human target genes were identified using TargetScanHuman database (release 8.0).⁹⁰ Functional annotation and enrichment analysis of the selected genes were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID),⁹¹ focusing on Gene Ontology Biological Process (GO BP) terms. The threshold for significance was set to p value ≤ 0.05 (EASE score) and Enriched GO BP terms were visualized through a bubble plot generated in R Studio (version 2025.05.1, Build 513) using the ggplot2 package.

Human monocyte transfection and osteoclastogenesis assessment

For CD14⁺ monocyte transfection, cells were seeded 625,000 cells/cm² in 96-well plates in α -MEM containing 10% FBS, 1% P-S, and 1% L-G supplemented with 25 ng/mL M-CSF (Myltenyi Biotec) and 50 ng/mL RANKL (PeproTech) and cultured for 3.5 days prior to transfection. miRNA complexes were formed through electrostatic interaction of RALA (GenScript)⁹² at an N:P ratio of 10⁹³ for miRNA mimic hsa-miR-150-5p and Mimic Negative Control #1 (scrambled control) (Horizon Discovery) at concentrations of 1 ng/ μ L and 5 ng/ μ L in OptiMEM (Gibco). Complexes were added to the cells following PBS wash and incubated for 5 h at 37°C. +RANKL positive control was incubated with OptiMEM only for 5 h at 37°C. At the end of the incubation, complexes were removed, and cells were cultured in the same media formulation as before transfection for an additional 3.5 days. At the end of culture, osteoclast differentiation was assessed by TRAP and DAPI staining as described above. Cells were imaged using Olympus IX83 inverted microscope with 10 $\times$ objective, and osteoclasts, number of nuclei per osteoclast, and total number of cells were manually counted using the cell counter plugin on ImageJ software (version 1.54p, NIH, USA).

RNA sequencing

RNA sequencing was performed following osteocyte-derived EV-F treatment or transfection with 5 ng/ μ L miR-150-5p under osteoclastogenic conditions (RANKL and M-CSF). For EV experiments, EV-F-treated osteoclast precursors were compared with cells exposed to RANKL and M-CSF alone (+RANKL), whereas for miRNA experiments, miR-150-5p transfected cells were compared with scrambled miRNA controls under the same conditions. Samples were sent to Novogene (United Kingdom) for sequencing analysis. Quality control of raw reads was carried out using FastQC.⁹⁴ Paired-end reads of 150 bp length were mapped against human assembly GRCh38 (hg38) with the short-read mapper STAR⁹⁵ and quantified using featureCounts.⁹⁶ Removal of lowly expressed genes was based on TPM values (transcripts per million). Genes that had a TPM of at least 2 in the majority of samples within a condition were included. Differential expression analysis was carried out with DESeq2.⁹⁷ For PCA, data were transformed using variance stabilizing transformation (vst) with DESeq2. PCA was performed using the R package PCAtools (version 2.20.0) and using all genes that passed the TPM cutoff ($n = 15,507$) but excluding the lowest 10% of variables based on variance. Multiple testing correction of p values was carried out using the Benjamini-Hochberg method. Differentially expressed genes (DEGs) were identified using an FDR < 0.05 and $|\log_2 \text{foldchange}| > 0.2$. Downregulated DEGs were intersected with known targets of miR-150-5p using the TargetScanHuman database (release 8.0).⁹⁰ GO enrichment analysis was carried out on DEGs that intersected with known miR-150-5p target genes using the enrichGO function from the clusterProfiler R package (version 4.16.0). Heatmaps and volcano plots were generated in R (version 4.5.0) with the packages ComplexHeatmap (version 2.24.1), ggplot2 (version 4.0.2), and ggrepel (version 0.9.7).

Statistical analysis

All data were analyzed using GraphPad Prism 10.2.2. All data were analyzed using an ordinary one-way or two-way ANOVA, assuming Gaussian distribution. Statistically significant differences were indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

DATA AND CODE AVAILABILITY

The data are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

M.M.: study design, investigation, data analysis, visualization, and writing – original draft. C.M.: contribution to investigation. R.A.: extracellular vesicle collection and characterization. S.P.: contribution to investigation. M.Y.B.: contribution to investigation and writing – review and editing. L.A.M.: SEM samples preparation and imaging. C.G.: extracellular vesicle collection. T.N.N.: contribution to investigation. C.T.B.: contribution to study design, technical support, and interpretation of data. F.M.R.:

formal analysis, bioinformatics analysis, and interpretation of RNA sequencing data. L.O.D.: contribution to study design, technical support, and interpretation of data. D.A.H.: conceptualization, study design, resources, writing – original draft and review and editing, supervision, project administration, and funding acquisition. All authors critically revised the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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