

Supplementary Material: ‘Educated’ Osteoblasts Reduce Osteoclastogenesis in a Bone-Tumor Mimetic Microenvironment

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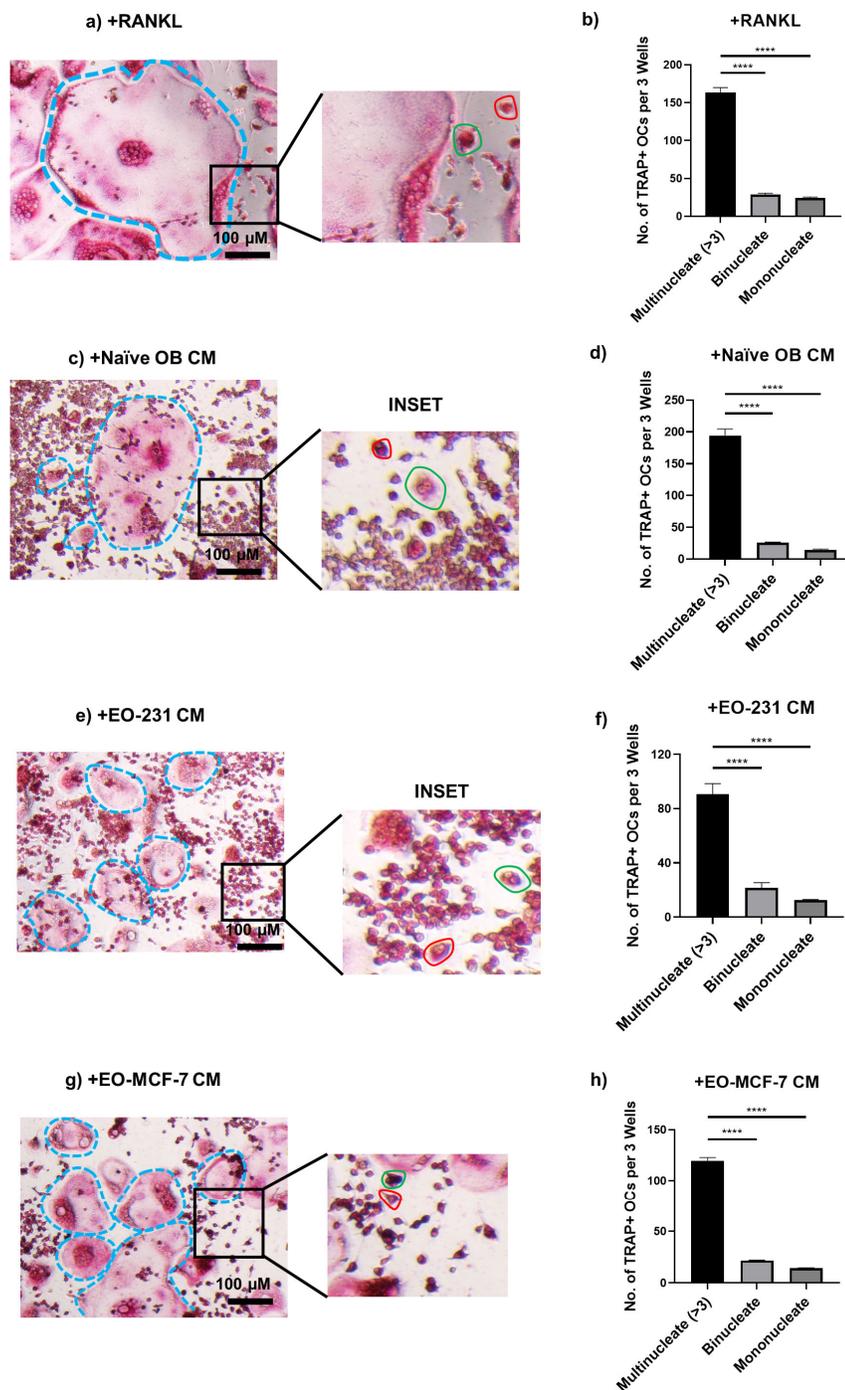


Figure 1. Multinucleated, TRAP-positive Osteoclasts are Greater in Number Compared to Binucleated and Mononucleated TRAP-positive Osteoclasts. RAW 264.7 pre-osteoclasts were cultured in the (a) presence of 50 ng/ml exogenous RANKL (control) and (b) the number (No.) of nuclei (multinucleated (≥ 3 nuclei), binucleated, and mononucleated) were quantified. Additionally, RAW 264.7 pre-osteoclasts were exposed to (c) naïve OB CM as an additional control and (d) the number (No.) of nuclei (multinucleated (≥ 3 nuclei), binucleated, and mononucleated) were quantified. For the first experimental condition, RAW 264.7 pre-osteoclasts were exposed to (e) EO-231 CM and (f) the number (No.) of nuclei (multinucleated (≥ 3 nuclei), binucleated, and mononucleated) were quantified. For the second experimental condition, RAW 264.7 pre-osteoclasts were exposed to (g) EO-MCF-7 CM and (g) the number (No.) of nuclei (multinucleated (≥ 3 nuclei), binucleated, and mononucleated) were quantified. All cultures were stained for tartrate-resistant acid phosphatase (TRAP; pink color), an established marker for mature osteoclasts. Blue, dashed outlines indicate TRAP-positive, multinucleated (≥ 3 nuclei) osteoclasts. Green outlines indicate TRAP-positive, bi-nucleated osteoclasts. Red outlines indicate TRAP-positive, mononucleated osteoclasts. Black boxes indicate inset location. Scale = 100 μ M. $N = 3$. ***** $p < 0.0001$.

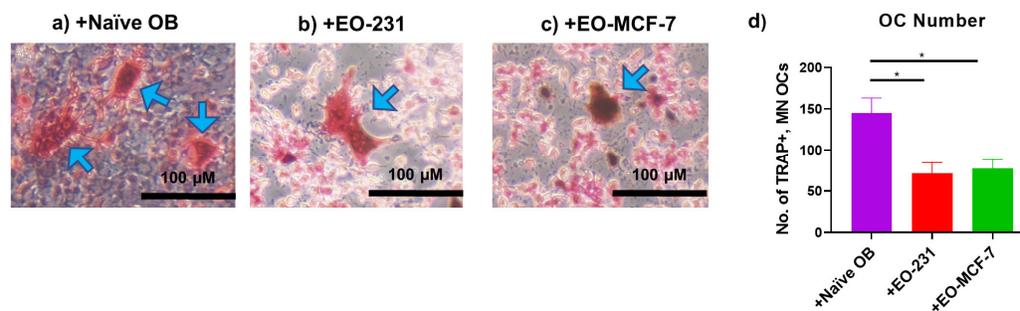


Figure S2. Primary Bone Marrow Monocytes Produced in the Presence of EO cells are Less in Number. CD11b+ primary bone marrow monocytes (BMMs) were collected as described in the Materials and Methods section. CD11b+ BMMs were co-cultured with (a) naïve OBs (control), (b) EO-231 cells, or (c) EO-MCF-7 cells in the presence of exogenous M-CSF and exogenous RANKL for 6 days. After 6 days, cultures were stained for tartrate-resistant acid phosphatase (TRAP; pink color), an established marker for mature osteoclasts. Blue arrows depict TRAP-positive, multinucleated (≥ 3 nuclei) osteoclasts. The number (No.) of TRAP-positive, multinucleated (≥ 3 nuclei) osteoclasts were quantified for each condition (d). Scale = 100 μ M. $N = 3$. * $p < 0.05$.

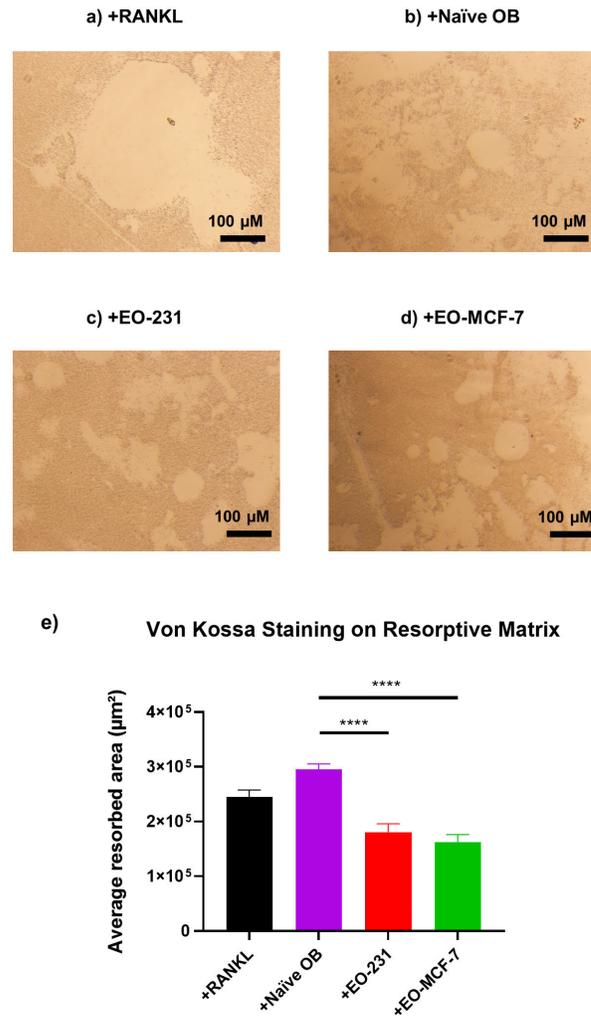


Figure S3. Modified Von Kossa Staining of Bone Mimetic Matrix. RAW 264.7 pre-osteoclasts were co-cultured on a bone mimetic matrix in the presence of 50 ng/ml exogenous RANKL (a) and co-cultured with either (b) naïve OBs (control), (c) EO-231 cells, or (d) EO-MCF-7 cells in the presence of 50 ng/ml exogenous RANKL for 6 days. A modified von Kossa staining was used to visualize resorptive pits. The average resorbed area was quantified for each condition (e). At least three individual batches were assayed per condition. Resorptive pits were visualized via light microscopy. Scale = 100 μM. $N = 3$. **** $p < 0.0001$.

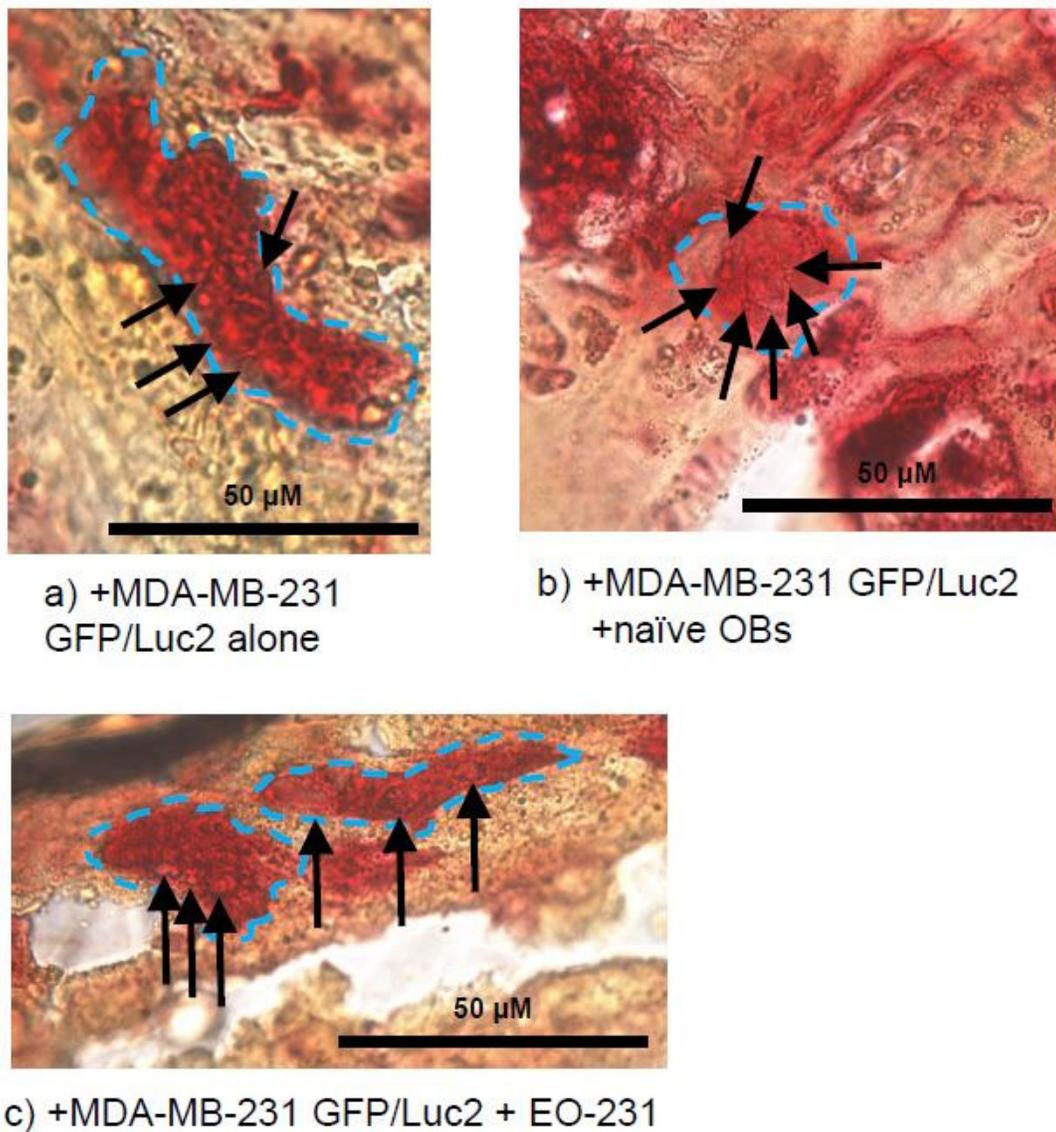


Figure S4. Multinucleated Osteoclasts are Found in the Bone-Tumor Microenvironment. Athymic nude mice were injected via intratibial injection with (a) MDA-MB-231GFP/Luc2 human breast cancer cells alone or (b) an admix of MDA-MB-231GFP/Luc2 human breast cancer cells plus naïve osteoblasts, or (c) an admix of MDA-MB-231GFP/Luc2 human breast cancer cells plus EO-231 cells. Eight weeks later, mice were euthanized and their tibia harvested. Tibia sections were stained for tartrate-resistant acid phosphatase (TRAP; blue dashed outlines), a common marker for mature osteoclasts. One representative image is displayed per condition. Black arrows indicate nuclei of TRAP-positive, multinucleated (≥ 3 nuclei) osteoclasts. Scale = 50 μ M.

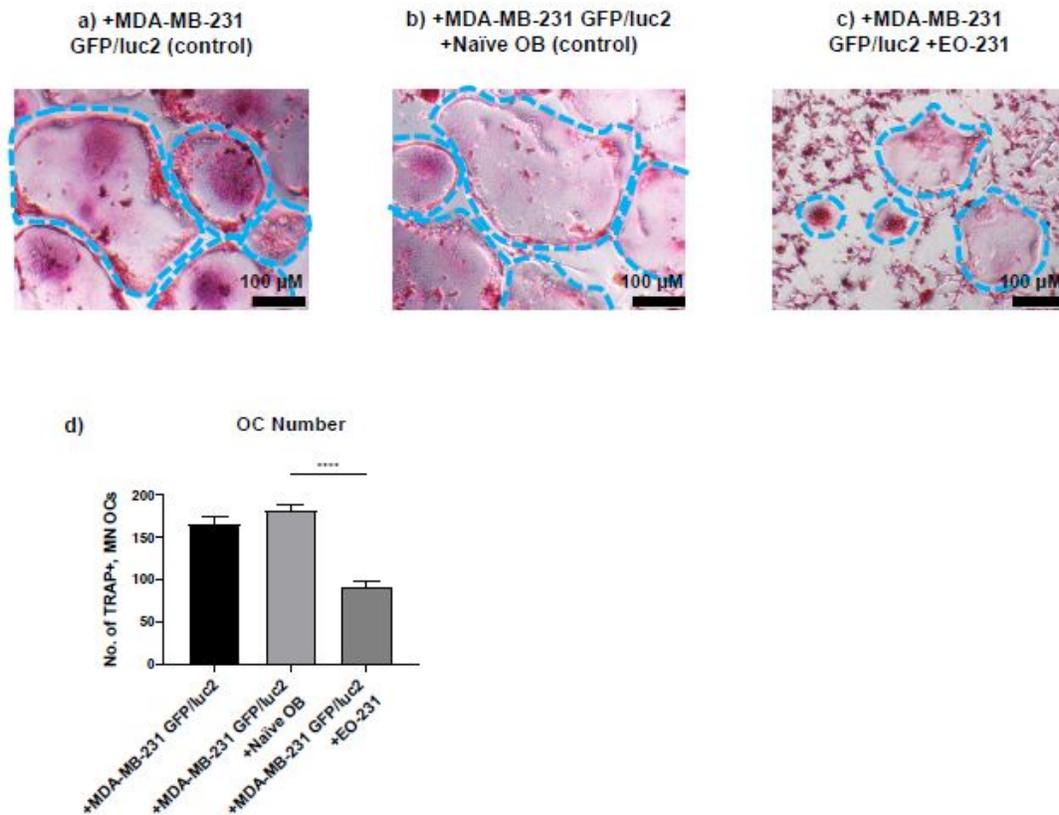


Figure S5. EOs Decrease the Number of TRAP-Positive, Multinucleated Osteoclasts in the Presence of GFP/luc2 MDA-MB-231 Human Breast Cancer Cells. RAW 264.7 pre-osteoclasts were cultured with (a) MDA-MB-231GFP/luc2 human breast cancer cells in the presence of 50 ng/ml exogenous RANKL alone for 6 days (control). RAW264.7 pre-osteoclasts were co-cultured with either (b) MDA-MB-231GFP/luc2 human breast cancer cells plus naïve OBs in the presence of 50 ng/ml exogenous RANKL (control) or (c) MDA-MB-231GFP/luc2 human breast cancer cells plus EO-231 cells in the presence of 50 ng/ml exogenous RANKL for 6 days. After 6 days, all conditions were stained for TRAP (blue outlines), a common marker for mature osteoclasts. The number (No.) of TRAP-positive, multinucleated (≥ 3 nuclei) mature osteoclasts were quantified for each condition (d). One representative image is shown per condition. Scale = 100 μ M. $N=3$. **** $p < 0.0001$.

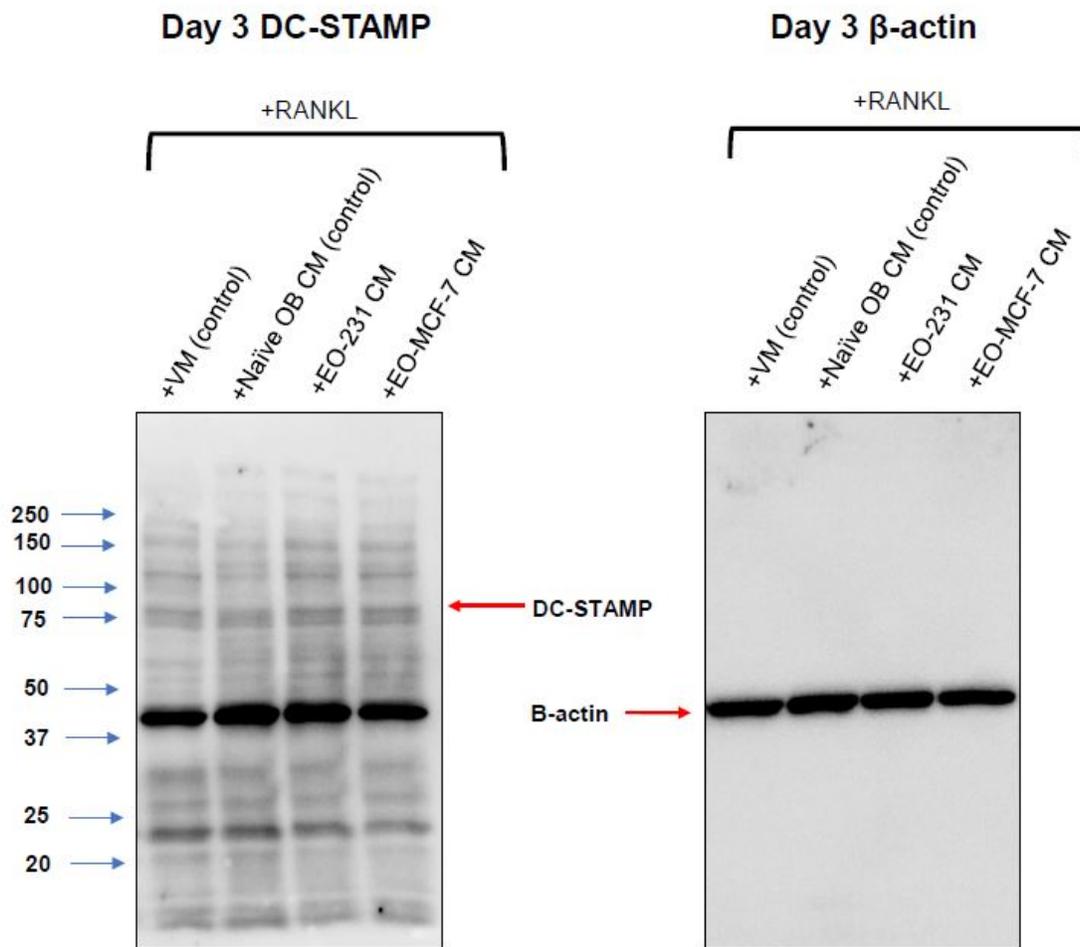


Figure 6. Western blot analysis of DC-STAMP and β -actin on Day 3 samples. Uncropped image used for Figure 6A. Samples order: Day 3 differentiated RAW 264.7 pre-OCs in vehicle media (VM); Day 3 differentiated RAW 264.7 pre-OCs exposed to naive OB CM; Day 3 differentiated RAW 264.7 pre-OCs exposed to EO-231 CM; Day 3 differentiated RAW 264.7 pre-OCs exposed to EO-MCF-7CM.

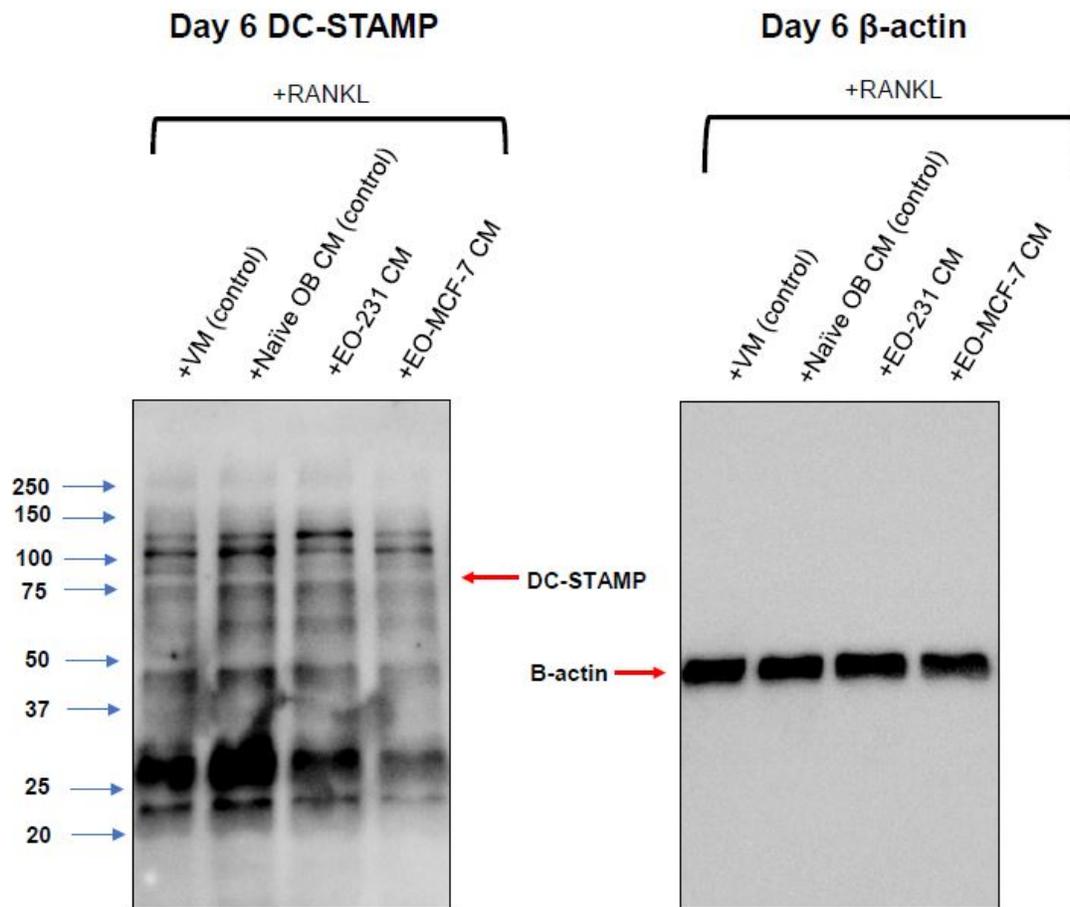


Figure S7. Western blot analysis of DC-STAMP and β -actin on Day 6 samples. Uncropped image used for Figure 6B. Samples order: Day 6 differentiated RAW 264.7 pre-OCs in vehicle media (VM); Day 6 differentiated RAW 264.7 pre-OCs exposed to naïve OB CM; Day 6 differentiated RAW 264.7 pre-OCs exposed to EO-231 CM; Day 6 differentiated RAW 264.7 pre-OCs exposed to EO-MCF-7 CM.

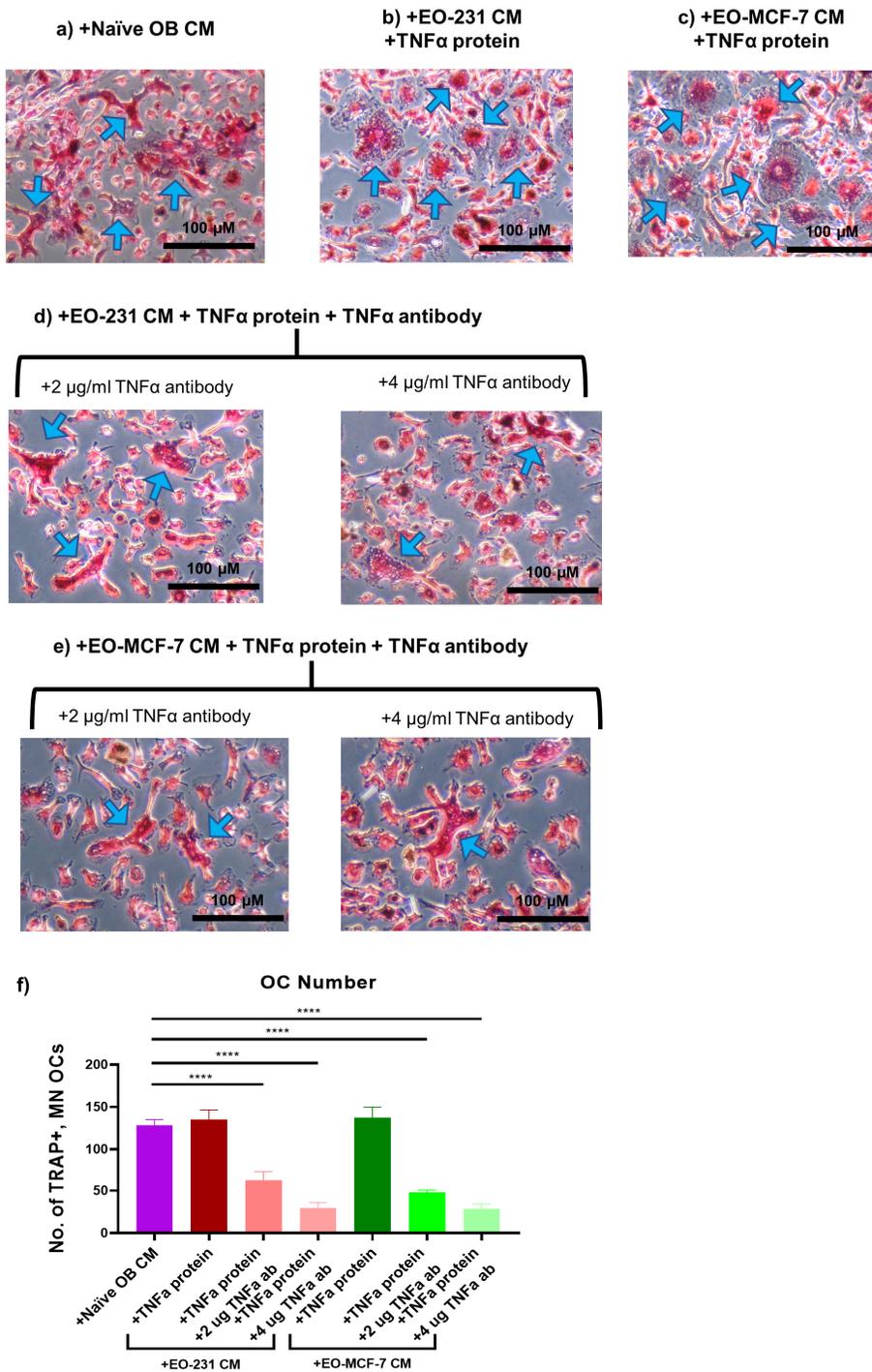


Figure S8. EO-mediated Osteoclast Formation using Primary Bone Marrow Monocytes can be Modulated by TNF α . CD11b⁺ primary bone marrow monocytes (BMMs) were collected as described in the Materials and Methods section. CD11b⁺ BMMs were exposed to (a) naïve OB CM (control), (b) EO-231 CM plus TNF α recombinant protein (12 ng/ml), (c) EO-MCF-7 CM plus Table 12. ng/ml), (d) EO-231 CM plus TNF α recombinant protein (12 ng/ml) plus TNF α neutralizing antibody (2 μ g/ml or 4 μ g/ml), or (e) EO-MCF-7CM plus TNF α recombinant protein (12 ng/ml) plus TNF α neutralizing antibody (2 μ g/ml or 4 μ g/ml). All cultures were maintained in the presence of exogenous M-CSF and exogenous RANKL for 6 days. After 6 days, cultures were stained for tartrate-resistant acid phosphatase (TRAP; pink color), an established marker for mature osteoclasts. Blue arrows depict TRAP-positive, multinucleated (≥ 3 nuclei) osteoclasts. The number (No.)

of TRAP-positive, multinucleated (≥ 3 nuclei) osteoclasts were quantified for each condition (f). Scale = 100 μM . $N = 3$. **** $p < 0.0001$.

Table 1. Estimation of Bone Resorptive Activity Per Individual Osteoclast.

Condition	Avg. Resorbed Area (μm^2)*	Avg. No. of TRAP-positive, multinucleated OCs [#]	Estimated Resorbed Activity by an Individual Osteoclast [†]
+RANKL	2706.29	173.75	15.57577
+Naïve OB CM	2452.91	312.2	7.856855
+EO-231 CM	1248.31	251.857	4.956424
+EO-MCF-7 CM	1038.25	192.429	5.395497

*avg resorbed area (μm^2) values for each condition as per Figure 3f. [#] avg number (No.) of TRAP-positive, multinucleated OCs values for each condition as per Figure 3e. [†] avg resorbed area/avg No. TRAP-positive, multinucleated OCs = estimated resorbed activity by an individual osteoclast RAW 264.7 pre-osteoclasts were co-cultured on a bone mimetic matrix with either naïve OBs (control), EO-231, or EO-MCF-7 in the presence of 50 ng/ml RANKL for 6 days. RAW 264.7 pre-osteoclasts on a mimetic bone matrix in the presence of 50 ng/ml RANKL for 6 days served as a control. The estimated resorptive activity of an individual osteoclast was quantified by dividing the average resorbed area by the average number of TRAP-positive, multinucleated osteoclasts (OCs) for each condition. The values for average resorbed area and the average number of osteoclasts for each condition were used from Figure 3f and Figure 3e, respectively.