Stromal-Derived Extracellular Vesicles Suppress Proliferation of Bone Metastatic Cancer Cells Mediated by ERK2

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ABSTRACT

Bone is a common site of cancer metastasis, including cancers such as breast, prostate, and multiple myeloma. Disseminated tumor cells (DTC) shed from a primary tumor may travel to bone and can survive undetected for years before proliferating to form overt metastatic lesions. This period of time can be defined as metastatic latency. Once in the metastatic microenvironment, DTCs engage in intercellular communication with surrounding stromal cells, which can influence cancer cell survival, proliferation, and ultimately disease progression. The role of the surrounding tumor microenvironment in regulating DTC fate is becoming increasingly recognized. We have previously shown that in the bone microenvironment, osteoblasts are “educated” by interactions with breast cancer cells, and these “educated” osteoblasts (EO) produce soluble factors that regulate cancer cell proliferation. In this study, we provide evidence indicating that EOs produce small extracellular vesicles (sEV) that suppress breast cancer proliferation, in part through regulation of ERK1/2 signaling. In addition, using EdU-incorporation assays and propidium iodide staining we demonstrate that exposure to EO-derived sEVs decreases breast cancer cell entry to S-phase of cell cycle. We also have evidence that particular microRNAs, including miR-148a-3p, are enriched in EO-derived sEVs, and that miR-148a-3p is capable of regulating breast cancer proliferation.

Introduction

Despite improvements in treatment, cancer patients that present with a primary tumor(s), including those with multiple myeloma, breast, or prostate cancer, may relapse and progress to metastatic disease. Bone, as a secondary site, is a common destination for cancer dissemination (1–3). We previously identified osteoblasts in the bone-tumor microenvironment as key regulators of breast cancer progression in bone (4–6). Furthermore, when in contact with metastatic breast cancer cells in vivo, we discovered an osteoblast subpopulation, defined as “educated” osteoblasts (EO), capable of suppressing breast cancer proliferation (5). Specifically, we found that soluble proteins in conditioned media (CM) from EOs, but not “uneducated” osteoblasts, reduced breast cancer proliferation (5).

Herein, we investigated intercellular communication between EOs and breast cancer cells. We hypothesized that EO-derived extracellular vesicles (EV) regulate breast cancer proliferation. EVs are implicated in a myriad of biological processes, and their role in cancer progression has been emphasized in recent years (7, 8). Cancer cell–derived EVs have been implicated in activation of cancer-associated fibroblasts (9, 10) and pre-metastatic niche formation (11, 12). Stromal cell–derived EVs are also capable of influencing cancer progression. In the bone microenvironment, it was shown that mesenchymal stem cells (MSC) produce EVs that suppress cancer cell proliferation (13, 14). Interestingly, the role of osteoblast-derived EVs in cancer progression in bone has not yet been investigated.

EVs are classified into subtypes, including exosomes (30–150 nm), microvesicles (100–1,000 nm), apoptotic bodies (<5,000 nm), among others, which differ in biogenesis and size (15, 16). Current separation techniques isolate heterogeneous EVs containing multiple subtypes; therefore, it is recommended to classify EVs according to size, density, or other characteristics (17). Here, we investigated small EVs (sEV), defined as <200 nm in diameter. sEVs from cancer cells and stromal cells play crucial roles in cancer progression and metastasis (7). Krishn and colleagues (18) demonstrated that prostate cancer–derived sEVs regulate endothelial cell activity and induce angiogenesis through sEV–mediated transfer of αvβ3 integrin. On the basis of this study and others that highlight the importance of sEVs in tumor progression, we decided to investigate sEVs in the bone-tumor microenvironment. Here, we demonstrate that EO-derived sEVs regulate breast cancer proliferation through modulation of ERK1/2 signaling. Furthermore, miR-148a-3p was upregulated in EO-derived sEVs, demonstrating that this microRNA regulates breast cancer proliferation, and...
modulates levels of ERK2 mRNA and protein. This may be one mechanism by which the bone microenvironment regulates progression of bone-metastatic breast cancer.

Materials and Methods

Detailed methods can be found in the Supplementary Materials and Methods, including Supplementary Tables S1 and S2.

Cells

Cells were cultured in humidified chamber at 37°C, 5% CO₂. MC3T3-E1 murine pre-osteoblasts gifted from Dr. Norman Karin, Roswell Park Cancer Institute (Buffalo, NY), were maintained in α-MEM (Gibco) plus 10% FBS (HyClone) and 100 U/mL penicillin 100 μg/mL streptomycin (Gibco), and were used passage ≤20 (19). MC3T3-E1 cells were differentiated using growth media containing 10 mM/L IL-6, 30 μg/mL Ascorbic acid (Sigma-Aldrich), EO-231 were used as previously described (5).

MDA-MB-231 cells were gifted from Dr. Danny Welch, Kansas University Medical Center (Kansas, KS), and were maintained in DMEM (Gibco), 5% FBS, and 100 U/mL penicillin 100 μg/mL streptomycin. For in vivo experiments, MDA-MB-231 cells expressing GFP and luciferase (pLeGo-IG2-Luc2 vector) were used, gifted from Dr. Alessandro Fatatis (Drexel University, Philadelphia, PA; ref. 20). MCF7 cells purchased from the ATCC were maintained in EMEM (Gibco), 10% FBS, 100 U/mL penicillin 100 μg/mL streptomycin, and 0.01 mg/mL recombinant human insulin (MP Biomedicals). All breast cancer cells were used within 20 passages between experiments.

Cells tested negative for Mycoplasma (Universal Mycoplasma Detection Kit, ATCC), which was last done March, 2021.

sEV isolation

sEVs (<200 nm) were isolated from culture media. Cells were grown to 60%–70% confluency in growth media (approximately 6.1 × 10⁶ cell/cm² for osteoblasts and EOs, and 8.7 × 10⁵ cells/cm² for breast cancer cells in T175 flasks). Cells were washed with 1X PBS, and media replaced with EV-depleted FBS media. After 48 hours, sEV-containing media were collected, and centrifuged at 1,000 x g for 5 minutes at 4°C to remove large debris. The sEV-containing media were filtered with 0.2-μm polyethersulfone filter and ultracentrifuged for 1 hour, 10 minutes at 120K x g at 4°C. After spun, supernatant was discarded, and pellet sEVs (in 1X PBS) were washed with 1X PBS, and centrifuged again at 4,000 x g for 30 minutes at 4°C. sEVs were isolated from culture media of EO-231, EO-MCF7, uneducated osteoblasts, and MDA-MB-231 cells. sEVs were characterized for expression of EV protein markers (CD63, CD9, and TSG101) compared with total cell lysates (Fig. 1A–C, Supplementary Fig. S1A and S1B; ref. 17). GM130, a golgi apparatus protein, or calnexin, an endoplasmic reticulum protein, served as negative controls and were not present in sEVs (Fig. 1A–C, Supplementary Fig. S1A and S1B; ref. 17). sEVs were analyzed using nanoparticle-tracking analysis (NTA), which indicated that sEV mode size was <200 nm and ranged from 97 to 110 nm in diameter (Fig. 1D). TEM imaging indicated sEVs from EO-231, uneducated osteoblasts, and MDA-MB-231 cells displayed standard morphology with no significant size differences (Fig. 1E).

In vitro chemotherapy experiments

MDA-MB-231 cancer cells were treated with vehicle (1X PBS), chemotherapeutic drug, sEVs, or pre-exposed to sEVs for 24 hours before drug treatment. MDA-MB-231 cells were plated (5 × 10⁴ cells) in growth media. 24 hours later (day 2), media were replaced with serum-free DMEM, and indicated wells pre-treated with EO-231 or uneducated osteoblast sEVs for 24 hours. On day 3, pre-treated cells were treated with doxorubicin (50 nmol/L) or paclitaxel (8 nmol/L) and other wells treated with EO-231 sEVs alone, uneducated osteoblast sEVs alone, doxorubicin alone, or paclitaxel alone. After 48 hours (day 5), media were refreshed, and fresh sEVs or drug added. 24 hours later (day 6) cells were counted. Three replicates were plated per condition, and experiment repeated four times.

Results

EOs produce sEVs

sEVs were isolated from culture media of EO-231, EO-MCF7, uneducated osteoblasts, as well as MDA-MB-231 and MCF7 breast cancer cells. sEVs were characterized for expression of EV protein markers (CD63, CD9, and TSG101) compared with total cell lysates (Fig. 1A–C, Supplementary Fig. S1A and S1B; ref. 17). GM130, a golgi apparatus protein, or calnexin, an endoplasmic reticulum protein, served as negative controls and were not present in sEVs (Fig. 1A–C, Supplementary Fig. S1A and S1B; ref. 17). sEVs were analyzed using nanoparticle-tracking analysis (NTA), which indicated that sEV mode size was <200 nm and ranged from 97 to 110 nm in diameter (Fig. 1D). TEM imaging indicated sEVs from EO-231, uneducated osteoblasts, and MDA-MB-231 cells displayed standard morphology with no significant size differences (Fig. 1E).

EO-derived sEVs decrease breast cancer proliferation

To determine the role of EO-derived sEVs in bone-metastatic breast cancer progression, we exposed triple-negative MDA-MB-231 cells to various concentrations of EO-231 sEVs and assessed proliferation via MTT assay. EO-231 sEVs decreased MDA-MB-231 proliferation in a concentration-dependent manner (Fig. 1F). Exposure to 20 μg/mL of EO-231 sEVs induced a change in MDA-MB-231 morphology and cells became rounded (Fig. 1F). Despite changes in proliferation and morphology, exposure to EO-231 sEVs did not affect MDA-MB-231 viability (Fig. 1F). Exposure to uneducated osteoblast-derived sEVs slightly decreased MDA-MB-231 proliferation, but did not alter cell morphology (Fig. 1G). EO-231 sEVs decreased MDA-MB-231 proliferation 6.7-fold at the highest concentration of sEVs used (20 μg/mL), whereas uneducated osteoblast sEVs decreased MDA-MB-231
Figure 1.  
EO-231 sEVs decrease MDA-MB-231 proliferation.  
A–C, Representative nanoparticle tracking analysis (NTA) and immunoblots for characteristic small EV protein markers in sEVs and total cell lysates (TCL) from (A) EO-231, (B) uneducated, differentiated osteoblasts (OB), and (C) MDA-MB-231 cells. CD63, CD9, and TSG101 are all characteristic EV markers. GM130 and calnexin served as negative controls that are not present in sEVs. GM130 is a golgi apparatus protein and calnexin is an endoplasmic reticulum protein.  
D, Comparison of concentration of sEVs produced by EO-231, EO-MCF7, and OBs. sEVs from different cell types were isolated from 72 mL of cell culture media and resuspended in 25 μL of 1X PBS. sEVs were diluted 1:250 before concentration was measured using NTA. Data represent mean particle concentration ± SEM.  
E, TEM images of sEVs from OBs, EO-231, and MDA-MB-231 cells; scale bar, 200 nm. Vesicle size from TEM images was quantified with ImageJ and data are depicted as box and whisker plot.  
F–H, MDA-MB-231 cells were exposed for 48 hours to different concentrations of sEVs or vehicle control (1X PBS) and proliferation was assessed with MTT assay. Data represent the mean percentage of proliferation compared with vehicle control ± SEM.  
I, MDA-MB-231 exposed to EO-231 sEVs. The top shows light microscopy images of MDA-MB-231 morphology following exposure to EO-231 sEVs. Bottom depicts live/dead assay of MDA-MB-231 cells exposed to EO-231 sEVs; scale bar, 100 μm.  
J, Ki67 quantification. Data represent the mean percentage of Ki67-positive cells after exposure to 20 μg/mL of OB sEVs, 20 μg/mL EO-231 sEVs, or vehicle control for 48 hours; Error bars, ± SEM, N = 3 replicates per condition; * P < 0.05, ** P < 0.001 for groups compared with vehicle control.
proliferation by only 1.5-fold (Fig. 1F and G). MDA-MB-231–derived sEVs did not alter MDA-MB-231 proliferation (Fig. 1H). Furthermore, exposure to EO-231 sEVs reduced MDA-MB-231 expression of Ki67 compared with cells exposed to vehicle (1.7-fold) or cells exposed to uneducated osteoblast sEVs (1.7-fold; Fig. 1I and J).

We additionally found that EO-MCF7 sEVs decreased ER+ MCF7 proliferation; however, at high concentrations, EO-MCF7 sEVs decreased MCF7 viability (Supplementary Fig. S1C). Uneducated osteoblast–derived sEVs or MCF7-derived sEVs did not alter MCF7 proliferation (Supplementary Fig. S1D and S1E).

**EO-derived sEVs reduce entry to S-phase of cell cycle**

We next assessed the effect of EO-derived sEVs on breast cancer cell cycle using an EdU assay. EO-derived sEVs reduced S-phase entry of MDA-MB-231 (1.7-fold) and MCF7 cells (1.5-fold; Fig. 2A and B). Uneducated osteoblast–derived sEVs did not alter S-phase entry of MDA-MB-231 or MCF7 cells (Fig. 2A and B). Per propidium iodide staining, exposure to EO-231 sEVs resulted in a 1.7-fold decrease in the percentage of MDA-MB-231 cells in S-phase compared with cells exposed to vehicle control or uneducated osteoblast sEVs (Fig. 2C). Interestingly, the effect of EO-derived sEVs on MDA-MB-231 and MCF7 proliferation was transient and removal of sEVs from culture media for 24 hours, restored breast cancer proliferation (Fig. 2D and E).

We repeated these experiments using sEVs isolated from primary murine osteoblasts and primary EO cells (Supplementary Fig. S2A–S2C). MDA-MB-231 cells exposed to primary EO sEVs exhibited decreased S-phase entry compared with cells exposed to vehicle control (2.4-fold) or cells exposed to primary osteoblast sEVs (1.9-fold; Supplementary Fig. S2D).

To validate whether the effect of EO sEVs on breast cancer proliferation was due to sEVs, as opposed to other soluble factors, MDA-MB-231 and MCF7 cells were exposed to EO sEVs, a comparable volume of EO CM (containing sEVs), or EO CM depleted of sEVs. CM was sEV-depleted by collecting the supernatant after the first ultracentrifugation step of the sEV isolation procedure (ultracentrifugation for 1 hour, 10 minutes at 120K x g). EO CM reduced S-phase entry of MDA-MB-231 (1.6-fold) and MCF7 cells (2.0-fold) compared with vehicle-treated cells (Supplementary Fig. S3A and S3B). Exposure to sEV-depleted EO CM resulted in a slight, but not significant, reduction in S-phase entry of MDA-MB-231 (1.2-fold) and MCF7 cells (1.3-fold) compared with vehicle-treated cells (Supplementary Fig. S3A and S3B). Comparatively, exposure to EO sEVs alone reduced S-phase entry of MDA-MB-231 (2.0-fold) and MCF7 cells (1.8-fold) relative to vehicle-treated cells (Supplementary Fig. S3A-B). In both breast cancer cell lines, there were no significant differences in S-phase entry between cells exposed to EO sEVs versus EO CM (Supplementary Fig. S3A and S3B). These data indicate that cancer cell exposure to EO CM decreases S-phase entry, but removal of sEVs from EO CM lessens this effect, thereby validating that sEVs present in CM are one key factor responsible for modulating cancer cell cycle.

**Density gradient-purified EO-derived sEVs potently regulate MDA-MB-231 proliferation**

Ultracentrifugation isolates heterogenous vesicles that may be contaminated by other particles, such as proteins and nucleic acids (17). To further purify sEVs, EO-231 sEVs were separated on an iodixanol density gradient. Enrichment of CD63, CD9, and TSG101 was observed in fractions with densities 1.117–1.160 g/L, with mode sizes 77–134 nm (Supplementary Fig. S3C and S3D).

sEVs from density fractions 1.117–1.160 g/L were pooled, and MDA-MB-231 cells exposed to 10 μg/mL of pooled iodixanol gradient EO-231 sEVs. Exposure to 10 μg/mL of pooled gradient EO-231 sEVs reduced MDA-MB-231 S-phase entry 1.5-fold compared with vehicle control (Supplementary Fig. S3E). Exposure to 20 μg/mL of pooled gradient EO-231 sEVs reduced MDA-MB-231 S-phase entry 2.6-fold compared with vehicle control (Supplementary Fig. S3E). This suggests that EO-derived sEVs, purified and isolated from an iodixanol density gradient, are potent regulators of breast cancer proliferation.

**EO-derived sEVs increase breast cancer cell expression of cell-cycle inhibitor proteins**

Because we observed that EO-derived sEVs altered breast cancer cell cycle, we investigated expression of cell-cycle inhibitor proteins, p21 and p27, following cancer cell exposure to EO-derived sEVs (21, 22). MDA-MB-231 cells exposed to EO-231 sEVs displayed increased levels of p21 and p27 (Fig. 3A and B). MDA-MB-231 cells exposed to uneducated osteoblast sEVs also had increased levels of p21 (3.1-fold) and p27 (8.9-fold); however, exposure to EO-231 sEVs led to greater increases in p21 (5.4-fold) and p27 (20.2-fold; Fig. 3A and B). In addition, p21 and p27 expressions increased in a dose-dependent manner based on sEV concentration (Fig. 3C and D). Similarly, MCF7 cells exposed to EO-MCF7 sEVs exhibited increased p21 levels compared with cells exposed to vehicle control or uneducated osteoblast sEVs (Fig. 3E). p27 was not significantly altered in MCF7 cells exposed to EO-MCF7 sEVs, but it was noted that control MCF7 cells expressed high amounts of p27 (Fig. 3F).

**EO-derived sEVs protect breast cancer cells from chemotherapy-induced cell death**

Because of the effect of EO-derived sEVs on breast cancer proliferation, we hypothesized that EO-derived sEVs may alter efficacy of chemotherapeutics. Chemotherapeutics, such as doxorubicin, target the DNA replication process that occurs during S-phase (23, 24). To test whether EO-derived sEVs protected breast cancer cells from chemotherapy-induced death, MDA-MB-231 cells were exposed to doxorubicin, EO-231 sEVs, or pre-exposed to EO-231 sEVs for 24 hours before doxorubicin treatment (Fig. 4A). Exposure to doxorubicin alone led to significantly less cells present compared with cells exposed to vehicle (Fig. 4B). Notably, there was no significant difference in the number of cells exposed to EO-231 sEVs alone versus cells pre-exposed to EO-231 sEVs before doxorubicin (Fig. 4B). Uneducated osteoblast sEVs did not have a protective effect on doxorubicin-induced cell death (Fig. 4B). A similar trend was observed when MDA-MB-231 cells were exposed to paclitaxel, a microtubule poison (25). There was no significant difference in cell number between cells exposed to EO-231 sEVs alone versus cells pre-exposed to EO-231 sEVs before paclitaxel (Fig. 4C). This is in contrast with cells exposed to vehicle control or uneducated osteoblast sEVs, which were not protected from paclitaxel-induced cell death (Fig. 4C).

To further investigate the effect of EO sEVs on chemotherapy-induced cell death, apoptosis was assessed via caspase activation assay (Fig. 4D). MDA-MB-231 cells pre-exposed to EO-231 sEVs before doxorubicin or paclitaxel exhibited decreased caspase 3/7 activation compared with cells exposed to chemotherapeutic drug alone, or cells pre-exposed to uneducated osteoblast sEVs before chemotherapy (Fig. 4D).

We hypothesized that EO sEVs exert a protective effect against chemotherapy-induced cell death, because chemotherapeutics, such as doxorubicin and paclitaxel, only target actively proliferating cells. Doxorubicin induces cell death by intercalating DNA and inhibiting
topoisomerase II, leading to DNA damage (24). To investigate the effect of EO sEVs on doxorubicin-induced DNA damage, levels of γH2AX, a marker of DNA double-strand breaks, were examined (26). Because of our hypothesis that EO sEVs protect against chemotherapy-induced death through reducing cancer cell proliferation, we simultaneously examined Ki67 expression. It was observed that EO-231 sEVs decreased MDA-MB-231 expression of Ki67 compared with MDA-MB-231 cells exposed to vehicle or uneducated osteoblast sEVs (Fig. 4E and F). This trend was maintained when cancer cells were exposed to doxorubicin (Fig. 4E and F). Furthermore, MDA-MB-231 cells pre-exposed to EO-231 sEVs before doxorubicin exhibited decreased γH2AX compared with cells pre-exposed to uneducated

Figure 2.
EO-derived sEVs decrease cancer cell entry to S-phase. A, MDA-MB-231 cells were exposed to 20 μg/mL EO-231 sEVs, uneducated osteoblast (OB) sEVs, or vehicle control (IX PBS) for 96 hours and proliferation was assessed via EdU assay. B, MCF7 cells were exposed to 18 μg/mL EO-MCF7 sEVs, OB sEVs, or vehicle control (IX PBS) for 96 hours and proliferation was assessed via EdU assay. Graphs represent the mean percentage of EdU-positive cells ± SEM. N = 3 replicates per condition; scale bars, 50 μm. **, P < 0.001. C, MDA-MB-231 cells were exposed to 20 μg/mL EO-231 sEVs, OB sEVs, or vehicle control (IX PBS) for 72 hours, and propidium iodide staining was used to assess cell-cycle distribution. Representative plots for each condition are shown. N = 3 replicates per condition. D, MDA-MB-231 cells were pre-exposed to 20 μg/mL EO-231 sEVs for 96 hours, then sEVs were removed and replaced with normal growth media for 24 hours, and an EdU assay was used to measure proliferation. E, MCF7 cells were pre-exposed to 18 μg/mL EO-MCF7 sEVs for 96 hours, then EVs were removed and replaced with normal growth media for 24 hours, and an EdU assay was used to measure proliferation. Bar graphs represent the mean percentage of EdU-positive cells ± SEM. N = 3 replicates per condition; scale bars, 50 μm; **, P < 0.001.
Figure 3.
EO-derived sEVs increase breast cancer cell expression of cell-cycle inhibitor proteins. A and B, MDA-MB-231 cells were exposed to 20 μg/mL EO-231 sEVs, uneducated osteoblast (OB)–derived sEVs, or vehicle control (1X PBS) for 96 hours, and levels of (A) p21 and (B) p27 were examined via immunofluorescence; scale bars, 50 μm. N ≥ 3 replicates per condition. #, P < 0.05; ##, P < 0.001. C and D, Immunofluorescent staining of (C) p21 and (D) p27 in MDA-MB-231 cells that were exposed for 96 hours to increasing amounts of EO-231 sEVs; scale bars, 50 μm. N ≥ 3 replicates per condition. #, P < 0.05 for groups compared with 0 μg/mL EO-231 sEV group (e.g., vehicle control). **#, P < 0.001 for groups compared with 0 μg/mL EO-231 sEV group (e.g., vehicle control). P, P < 0.05 for groups compared with 20 μg/mL EO-231 sEV group. E and F, MCF7 cells exposed to 18 μg/mL EO-MCF7 sEVs, OB-derived sEVs, or vehicle control (1X PBS) for 96 hours, and levels of (E) p21 and (F) p27 were examined via immunofluorescence; scale bars, 50 μm. N ≥ 3 replicates per condition. *, P < 0.05; **, P < 0.001. All bar graphs depict mean ± SEM.
Figure 4.
EO-derived sEVs protect breast cancer cells from chemotherapy-induced cell death. **A**, MDA-MB-231 cells were treated with either vehicle control (1X PBS), chemotherapeutic drug (doxorubicin or paclitaxel), 20 µg/mL EO-231-derived sEVs or uneducated osteoblast (OB) sEVs, or pre-exposed to 20 µg/mL EO-231-derived sEVs or OB sEVs for 24 hours before drug treatment. Cells were incubated in each condition as depicted in schematic (A), and the number of cells per condition was quantified by manually counting cells under light microscopy. **B**, Mean (±SEM) number of cells exposed to sEVs, doxorubicin, or a combination of sEVs and doxorubicin at day 6. **C**, Mean (±SEM) number of cells exposed to sEVs, paclitaxel, or a combination of sEVs and paclitaxel at day 6. **D**, Caspase 3/7 activation in MDA-MB-231 cells exposed to EO-231 sEVs or uneducated OB sEVs alone, or pre-exposed to sEVs for 24 hours before being subsequently exposed to chemotherapeutic agent for 48 hours. Graph represents the mean percentage of cells with active caspase 3/7 ± SEM. **E–G**, Ki67 and γH2AX levels in MDA-MB-231 cells exposed to 20 µg/mL EO-231 sEVs alone, 20 µg/mL uneducated OB sEVs alone, or pre-exposed sEVs for 24 hours before being subsequently exposed to doxorubicin for 24 hours. **F**, Representative immunofluorescent images from N ≥ 3 independent replicates; scale bars, 50 µm. ***, P < 0.001; ns, not significant. All experiments, N ≥ 3 replicates per condition.
osteoblast sEVs before doxorubicin or cells exposed to doxorubicin alone (Fig. 4F and G). This experiment was also carried out using paclitaxel; however, we found that paclitaxel did not induce pronounced DNA damage in MDA-MB-231 cells (Supplementary Fig. S4).

Ki67 and doxorubicin-induced DNA damage were also examined following pre-exposure to primary EO sEVs (Supplementary Fig. SSA-C). Primary EO sEVs decreased MDA-MB-231 expression of Ki67 expression compared with cells exposed to vehicle control or primary uneducated osteoblast sEVs (Supplementary Fig. SSA and SC). Moreover, pre-exposure to primary EO sEVs before doxorubicin resulted in decreased γH2AX compared with cells exposed to doxorubicin alone or cells pre-exposed to primary uneducated osteoblast sEVs before doxorubicin (Supplementary Fig. SSB and SSC).

EO-derived sEVs regulate ERK1/2 signaling in breast cancer cells

To determine the effect of EO-derived sEVs on proliferative signaling pathways, a Phospho-Kinase Array was carried out on MDA-MB-231 cells exposed to EO-231 sEVs. This array revealed decreased phosphorylation of proteins, including WNK1 (0.29-fold) and ERK1/2 (0.47-fold), following MDA-MB-231 exposure to EO-231 sEVs, compared with MDA-MB-231 cells exposed to uneducated osteoblast sEVs (Fig. 5A and B). In comparison with vehicle-treated cells, WNK1 phosphorylation was increased in MDA-MB-231 cells exposed to uneducated osteoblast sEVs, but not largely increased in cells exposed to EO-231 sEVs. WNK1 is involved in ion transport, and has also been implicated in angiogenesis (27). We chose to further investigate ERK1/2 signaling in breast cancer cells following sEV exposure, because ERK1/2 signaling (Fig. 5C) is a well-known direct driver of cell proliferation, and literature suggests that reduced ERK1/2 signaling is associated with decreased breast cancer proliferation and cell-cycle arrest (28, 29). In addition, elevated ERK1/2 phosphorylation was consistent between MDA-MB-231 cells exposed to vehicle control and uneducated osteoblast sEVs, whereas low phosphorylation was observed in MDA-MB-231 cells exposed to EO-231 sEVs, suggesting that modulation of ERK1/2 phosphorylation is specific to EO sEV exposure.

We further examined the ERK1/2 pathway and found that MDA-MB-231 cells exposed to EO-231 sEVs exhibited decreased phospho-ERK1/2, total ERK1/2, and phospho-p90RSK, a downstream target of ERK1/2 (Fig. 5D). Specifically, there was a large decrease in MDA-MB-231 levels of total ERK2 following exposure to EO-231 sEVs compared with cells exposed to vehicle or uneducated osteoblast sEVs (Fig. 5D). Similarly, exposure to EO-MCF7 sEVs resulted in decreased phospho-ERK1/2 and total ERK2, compared with MCF7 cells exposed to vehicle control or uneducated osteoblast sEVs (Fig. 5E). Of note, MCF7 cells did not express detectable amounts of phospho-p90RSK (data not shown). The effect of sEVs isolated from primary EOs on ERK1/2 signaling was also examined. Exposure to primary EO sEVs led to substantial reduction in phospho-ERK1/2 and minor reduction in total ERK2 (Fig. 5F). Although EO sEVs reduced MDA-MB-231 levels of phospho-ERK1/2 and total ERK2, this did not correspond with changes in active, phospho-MEK1/2 (Fig. 5D–F). MCF7 cells, however, exhibited decreased phospho-MEK1/2 following exposure to EO-MCF7 sEVs (Fig. 5E).

Breast cancer cells have altered ERK1/2 levels in intratibial tumors that include EO cells

To determine the effect of EOs on ERK1/2 signaling in the bone-tumor microenvironment, we injected an admix of MDA-MB-231GFP/Luc2 plus EO-231 into the tibiae of mice. MDA-MB-231GFP/Luc2 cells inoculated alone and MDA-MB-231GFP/Luc2 cells admixed with uneducated osteoblasts served as controls. After 8 weeks, tibiae were harvested and levels of phospho-ERK1/2 and total ERK1/2 were examined via immunofluorescence. In bones of tumor-bearing mice injected with MDA-MB-231GFP/Luc2 cells alone, there were high levels of phospho-ERK1/2 and total ERK1/2 in cancer cells (Fig. 6A; Supplementary Fig. S6). Similar high levels of phospho-ERK1/2 and total ERK1/2 were observed in cancer cells from bones injected with MDA-MB-231GFP/Luc2 plus uneducated osteoblasts (Fig. 6B; Supplementary Fig. S7). Strikingly, in bones of tumor-bearing mice injected with MDA-MB-231GFP/Luc2 plus EO-231, there were low levels of both phospho-ERK1/2 and total ERK1/2 (Fig. 6C, Supplementary Fig. S8).

We previously reported that expression of IL-6 and RUNX2 distinguished uneducated osteoblasts from EOs (5). Using these markers, we found uneducated osteoblasts and EOs in serial sections of tumorbearing bones injected with MDA-MB-231GFP/Luc2 cells alone, MDA-MB-231GFP/Luc2 cells plus uneducated osteoblasts, and MDA-MB-231GFP/Luc2 cells plus EO-231 (Fig. 6, Supplementary Figs. S6–S8).

ERK2 regulates MDA-MB-231 proliferation and chemotherapy sensitivity

Because we observed that EO-derived sEVs decreased cancer cell levels of ERK2, we examined the role of ERK2 versus ERK1 in regulating breast cancer proliferation. MDA-MB-231 cells were transiently transfected with siRNAs against ERK2, ERK1, or scrambled siRNA control (Supplementary Fig. S9A). Knockdown of ERK2, but not ERK1, resulted in a decrease in S-phase entry (Supplementary Fig. S9B and S9C). Interestingly, knockdown of ERK2 or ERK1 in MDA-MB-231 cells led to increases in p27, but not p21 (Supplementary Figs. S9D–S9G). This suggests that although ERK1 and ERK2 may both regulate expression of p27, ERK2 may play a larger role in promoting MDA-MB-231 proliferation.

Because of the observation that EO-derived sEVs alter ERK1/2 expression in cancer cells, we hypothesized that reduction of ERK1/2 signaling may be a mechanism by which EO-derived sEVs protect breast cancer cells from chemotherapy-induced death. To test this, MDA-MB-231 cells were transiently transfected with siRNAs against ERK2 or ERK2 before being exposed to doxorubicin (Supplementary Fig. S10A). MDA-MB-231 cells knocked-down for ERK2 exhibited decreased cell death following doxorubicin exposure compared with cells transfected with scrambled siRNAs (Supplementary Fig. S10B–S10D). Knockdown of ERK1 did not exert a protective effect against doxorubicin (Supplementary Fig. S10E and S10F), which is consistent with data demonstrating that knockdown of ERK1 does not alter MDA-MB-231 proliferation (Supplementary Fig. S9C).

miR-148a-3p is upregulated in EO-231 sEVs and regulates breast cancer proliferation

Previous studies have demonstrated an important role for sEV-associated microRNAs in cancer progression in the tumor microenvironment. Bliss and colleagues (13) reported that sEV-associated microRNAs from MSCs induced breast cancer cell-cycle arrest. To determine whether sEV-associated microRNAs play a role in regulating breast cancer proliferation in our system, we carried out a targeted array to examine microRNAs present in EO and uneducated osteoblast-derived sEVs. We discovered several microRNAs differentially present in sEVs from EO-231 cells compared with uneducated osteoblasts (Fig. 7A). A literature search of the top 10 most up- and
downregulated microRNAs was carried out to identify microRNAs associated with cell proliferation. Compared with other microRNAs discovered through the array, miR-148a-3p emerged as a promising target, due to previous literature indicating it is a tumor-suppressive microRNA that decreases cell proliferation in breast, colorectal, and pancreatic cancers (30–33). In addition, miR-148a-3p has been linked to suppression of metastasis in multiple studies (34, 35). Furthermore, it has been shown in endometrial cancer, that tumor-promoting cancer associated fibroblasts have low levels of miR-148a-3p (36). We further investigated miR-148a-3p, which has an identical sequence in mice and humans, using Tarbase, miRTarBase, and TargetScan (37–39). This search revealed that miR-148a-3p is predicted to target several genes involved in proliferation and cancer progression, including ERK2. Because of the role of miR-148a-3p in regulating cancer proliferation, the link of miR-148a-3p and metastasis suppression, and the potential regulation of ERK2, we chose to follow-up on miR-148a-3p as a potential mechanism responsible for the effect of EO sEVs on cancer cell proliferation.

Figure 5.
Exposure to EO-derived sEVs alters ERK1/2 signaling in breast cancer cells. A, Phospho-Kinase Array of MDA-MB-231 cells exposed vehicle control (1X PBS), or 20 μg/mL EO-231-derived sEVs, or 20 μg/mL uneducated osteoblast (OB) sEVs for 48 hours. B, Signal intensity was quantified as fold change in MDA-MB-231 cells exposed to EO-231 sEVs compared with cells exposed to OB sEVs. C, Schematic depicting ERK1/2 signaling pathway. D, Immunoblot of proteins in the ERK1/2 signaling pathway from MDA-MB-231 cells that were exposed to 20 μg/mL EO-231 sEVs, OB sEVs, or vehicle control (1X PBS) for 72 hours. E, Immunoblot of proteins in the ERK1/2 signaling pathway from MCF7 cells that were exposed to 18 μg/mL EO-MCF7 sEVs, OB sEVs, or vehicle control (1X PBS) for 72 hours. F, Immunoblot of proteins in the ERK1/2 signaling pathway from MDA-MB-231 cells that were exposed to 20 μg/mL primary EO sEVs, primary OB sEVs, or vehicle control (1X PBS) for 72 hours. For all blots, the densitometry values, which were normalized to actin protein expression, are depicted below each blot.
To determine the role of miR-148a-3p in regulating breast cancer cell proliferation, MDA-MB-231 and MCF7 cells were transiently transfected with a miR-148a-3p mimic. Transfection with miR-148a-3p mimic decreased S-phase entry of MDA-MB-231 and MCF7 cells compared with cells transfected with scrambled control (Fig. 7B and C). In addition, transfection with miR-148a-3p mimic increased p27, but not p21, in MDA-MB-231, and MCF7 cells (Supplementary Fig. S11).

To determine whether sEV-associated miR-148a-3p is responsible for decreased breast cancer cell proliferation, we transfected MDA-MB-231 cells with a miR-148a-3p inhibitor or scrambled control. Breast cancer cells were exposed to EO-231–derived sEVs for 48 hours, and EdU used to assess proliferation. Cells transfected with scrambled miR control exhibited decreased proliferation upon exposure to EO-231 sEVs for 48 hours (Fig. 7D). Conversely, when cells were transfected with an inhibitor against miR-148a-3p, cell proliferation was not significantly altered following exposure to EO-231 sEVs (Fig. 7D).

We next examined the effect of miR-148a-3p on ERK2 expression. MDA-MB-231 cells transiently transfected with miR-148a-3p mimic

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**Figure 6.**
Expression of phospho-ERK1/2 and total ERK1/2 in the endosteal niche. Athymic nude mice were injected via intratibial injection with (A) MDA-MB-231GFP/Luc2 breast cancer cells alone, (B) MDA-MB-231GFP/Luc2 cells admixed with MC3T3-E1 osteoblasts (OB), or (C) MDA-MB-231GFP/Luc2 cells admixed with EO-231 cells. Eight weeks later, mice were euthanized and the tibiae were harvested. Bone tissue sections were stained for phospho-ERK1/2 (pERK1/2) and GFP, or total ERK1/2 and GFP to identify cancer cells expressing ERK1/2. Stained sections were analyzed via immunofluorescence microscopy. In each condition, the top row of images depicts the bone/tumor interface, whereas the lower row of images include regions composed of tumor cells alone. These areas were within the same bone section. Serial sections were stained for osteoblast markers (RUNX2 and IL-6). Osteoblasts are indicated by yellow arrows. EO-231 cells were identified by staining RUNX2 positive and IL-6 negative. EO cells are indicated by white arrows. As shown on the tibia at the left, the black box represents the locations in the bone where the images were taken. At least three independent, serial sections were stained per bone and three bones examined per condition. Shown are representative images of n = 3 mice per condition; scale bar, 50 μm.
Figure 7.
EO-derived sEVs contain miR-148a-3p that regulates breast cancer cell proliferation. A, Relative levels of the top 10 most upregulated and 10 most downregulated microRNAs in sEVs from EO-231 compared with sEVs from uneducated osteoblasts. B, MDA-MB-231 cells or (C) MCF7 cells were transiently transfected with a miR-148a-3p mimic or scrambled miR control and S-phase entry was assessed via EdU-incorporation assay. **, P < 0.001. D, MDA-MB-231 cells were transiently transfected with a scrambled miR control or miR-148a-3p inhibitor before being exposed to 20 μg/mL EO-231 sEVs for 48 hours. An EdU assay was used to assess S-phase entry. Data represent mean percentage of EdU-positive cells ±SEM. N = 3 replicates per condition. **, P < 0.001; ns, not significant. E, MDA-MB-231 cells were transiently transfected with a scrambled miR control, miR-148a-3p mimic, or a miR-148a-3p inhibitor, and levels of the ERK2 transcript were then measured via qPCR. Relative quantification of N = 3 replicates ±SEM is shown. **, P < 0.001; ns, not significant. F, Immunoblot of MDA-MB-231 cells transfected with a scrambled miR control, miR-148a-3p mimic, or miR-148a-3p inhibitor. Levels of phospho-ERK1/2 and total ERK1/2 were examined. Densitometry values, normalized to actin, are indicated below blot. G, Schematic of EO-derived sEV communication with breast cancer cells.
had decreased ERK2 transcript levels compared with cells transfected with scrambled control (Fig. 7E). No significant changes in ERK2 expression were observed in cells transfected with miR-148a-3p inhibitor versus cells transfected with scrambled control. Transfection with a miR-148a-3p mimic also decreased MDA-MB-231 levels of ERK2 protein compared with cells transfected with scrambled control (Fig. 7F). Levels of ERK1, as well as phospho-ERK1/2, were largely unchanged by the miR-148a-3p mimic (Fig. 7F). These data suggest that miR-148a-3p, which is present in EO-231 sEVs, is a regulator of breast cancer proliferation, and may exert its effect in part through modulation of ERK2.

**Discussion**

We previously showed that osteoblasts are “educated” by interactions with breast cancer cells, and EOs produce soluble proteins that regulate breast cancer proliferation (5, 6). Here, we demonstrate that EO-derived sEVs are another mechanism regulating cancer cell proliferation (Fig. 7G). We characterized sEVs from EOs, uneducated osteoblasts, and breast cancer cells, and observed similar size and protein marker expression between sEVs isolated from these various cell types (Fig. 1A–C, Supplementary Figs. S1A–S1B and S2B–S2C). We also observed that EO-231, EO-MCF7, and uneducated osteoblasts produced similar numbers of sEVs (Fig. 1D). A study by Dourado and colleagues (40) reported that cancer-associated fibroblasts produced more EVs than normal fibroblasts, suggesting that interactions with cancer cells can enhance EV secretion. However, in our study, cancer cell “education” of osteoblasts did not alter sEV production.

We found that EO-derived sEVs reduced cancer cell S-phase entry by approximately 40% (Fig. 2A–C, Supplementary Fig. S2D). This indicates that although EO sEVs suppress breast cancer cell proliferation, this effect is only observed in a subset of cancer cells. In addition, it was observed that uneducated osteoblast-derived sEVs had a small effect on cancer cell proliferation and expression of cell-cycle inhibitor proteins (Figs. 1G, 3A and B); however, it was observed that the effect of EO-derived sEVs on cancer cells was more profound. For example, using an MTT assay it was found that exposure to 20 μg/mL uneducated osteoblast-derived sEVs decreased MDA-MB-231 proliferation 1.5-fold compared with vehicle control, whereas exposure to 20 μg/mL EO-231 sEVs decreased MDA-MB-231 proliferation 6.7-fold compared with vehicle control (Fig. 1F and G). Although we observed that MDA-MB-231 cells may be sensitive to uneducated osteoblast sEVs with regards to proliferation and cell-cycle inhibitor protein expression, MCF7 cells were not (Fig. 3E; Supplementary Fig. S1). MDA-MB-231 cells are triple negative, whereas MCF7 cells are ER+; which may contribute to different responses observed in this study; however, the exact mechanism underlying the differential responses is currently unknown.

We found EO sEVs to be protective against chemotherapy-induced death (Fig. 4A–D). Specifically, pre-exposure to EO sEVs prevented chemotherapy-induced apoptosis and doxorubicin-induced γH2AX (Fig. 4D–F). The presence of γH2AX was also examined following paclitaxel exposure; however, we did not observe paclitaxel to induce DNA damage in this system. Paclitaxel inhibits microtubules, which is a distinct mechanism of action compared with doxorubicin, and may account for why strong DNA damage was not observed following MDA-MB-231 exposure to paclitaxel (24, 25). Future investigations are planned to investigate the role of EO sEVs in DNA damage repair. Overall, these data suggest that EOs suppress breast cancer proliferation, which may in turn protect cancer cells from chemotherapy-induced death in the bone microenvironment. In addition, these findings raise questions about what occurs during cancer progression in bone that induces non-proliferative cancer cells to form overt metastases. It could be the case that over time the abundance or content of EO-derived sEVs is changed, which may influence cancer progression. It is also possible that the number of EOs in the bone-tumor microenvironment decreases over time, as has previously been observed with osteoblasts, which may allow for metastatic lesion outgrowth (41).

The role of the tumor microenvironment in regulating cancer progression is becoming increasingly evident. In bone, several studies have indicated that osteoblasts have tumor-suppressive or dormancy-inducing properties. In prostate cancer, it was reported that GAS6 produced by osteoblasts interacts with AXL on the cancer cell surface to induce prostate cancer cell-cycle arrest and chemotherapy resistance (42, 43). In a separate study, it was found that osteoblasts secrete GDF10 and TGFβ2, which activated the p38 signaling pathway to suppress prostate cancer proliferation (44). Our data further support these studies by demonstrating that EOs, an osteoblast subpopulation, engage in sEV-mediated communication with breast cancer cells to regulate cancer proliferation and chemotherapy sensitivity. In addition, our data may help explain how some cancer cells persist in the bone after a patient has undergone chemotherapy treatments. A recent study by Loftus and colleagues (45) investigated crosstalk between osteoblasts and cancer cells, and found that breast cancer-derived factors induced naïve osteoblasts to secrete cytokines and EVs with pro-inflammatory and pro-osteoclastogenic functions. The authors found exposure to MDA-MB-231–derived EVs for 48 hours, increased osteoblast expression of Rankl, Il6, and Il1b, among other cytokines (45). Previously, our group found that EOs, an osteoblast subpopulation with antitumor properties, express low levels of inflammatory cytokines, which supports our proposed tumor-inhibitory role of EOs in cancer progression (5). These two studies highlight the complexity of tumor–stroma crosstalk in metastatic progression, and suggest distinct events in early- versus late-stage disease. To illustrate this point, work from our group indicates that EOs reduce osteoclastogenesis via alterations in TNFα expression (46). Additional studies investigating EOs throughout cancer progression are currently ongoing. It is likely that EOs will uniquely interact with other cells in the microenvironment. The study here specifically focused on the effect of sEVs derived from EOs and their effect on cancer cell proliferation.

Several studies have reported the presence of specific markers to identify non-proliferative disseminated tumor cells (DTC; ref. 47). These markers include high levels of the cell-cycle inhibitors, p21 and p27, and low levels of Ki67 (42, 44, 47, 48). We found that exposure to EO-derived sEVs reduced breast cancer cell expression of Ki67 (Fig. 1I and J) and increased p21 and p27 (Fig. 3G). p21 and p27 inhibit multiple cyclin–CDK complexes at different stages of the cell cycle (49, 50). We observed that EO-derived sEVs reduce breast cancer cell entry to S-phase, which is in accordance with our data demonstrating that EO-derived sEVs increase breast cancer cell levels of p21 and p27. Furthermore, our findings support previous studies that propose low Ki67 and high p21 and p27 as markers of non-proliferative cancer cells (42, 44, 47, 48).

We identified that ERK1/2 signaling was altered in breast cancer cells exposed to EO-derived sEVs (Fig. 5). Knockdown of ERK2, but not ERK1, decreased proliferation of MDA-MB-231 cells (Supplementary Fig. S9B–S9C), but knockdown of both ERK2 or ERK1 increased MDA-MB-231 levels of p27 (Supplementary Fig. S9D–E). Levels of p21 were unchanged in cancer cells following knockdown of ERK2 or ERK1 (Supplementary Fig. S9F and S9G). This suggests that EO-derived sEVs may alter other pathways, in addition to ERK1/2,
that transfection with a miR-148a-3p mimic actually increased miR-148a-3p (p27) transcript (39). Despite the fact that there is a potential miR-148a-3p-binding site in CDKN1B, in our study we observed that transfection with a miR-148a-3p mimic actually increased cancer cell levels of p27 protein (Supplementary Fig. S11A and S11B). It is possible that this mechanism is linked to ERK1/2 regulation of p27, as we also observed that knockdown of ERK1/2 led to increased p27 levels (Supplementary Fig. S9D-E). It may also be possible that miR-148a-3p regulation of p27 occurs through an ERK1/2-independent mechanism. Furthermore, the observation that transfection with a miR-148a-3p mimic did not alter breast cancer levels of p21 (Supplementary Fig. S11C and S11D) suggests that there may be other EO-derived factors, in addition to miR-148a-3p, that increase p21 expression, as was observed when cancer cells were exposed to EO sEVs (Fig. 3).

Our data provide insight into sEV-mediated communication between EOs and breast cancer cells; however, there are limitations to this study. Currently, the physiological abundance of EO-derived sEVs in the bone-tumor microenvironment is unknown. We found that EO-derived sEVs require concentrations around 18–20 μg/mL to potently suppress breast cancer proliferation (Fig. 1A; Supplementary Fig. S1C). Similar concentrations of sEVs were reported by Krishn and colleagues (18), whereby 20–40 μg/mL of prostate cancer sEVs were needed to elicit a functional effect. Another limitation of our study is we only investigated the role of sEV-associated miR-148a-3p in regulating breast cancer proliferation. It is evident that other micro-RNAs, proteins, DNA, and/or lipids may be important sEV cargo that could regulate cancer proliferation. In addition, we investigated the effect of EO sEVs on ERK1/2; however, our data indicate that EO sEVs alter other proliferation-associated pathways that could be avenues for future investigation (Fig. 5A and B). For example, decreased STAT3 phosphorylation was observed in MDA-MB-231 cells exposed to EO sEVs, compared with cells exposed to uneducated osteoblast sEVs. STAT3 is known to promote cell proliferation and tumorigenesis (60). Therefore, decreased STAT3 activation may be another mechanism that suppresses cancer proliferation, which was not examined in this study. Another area of investigation not explored in this study is the effect of EO sEVs on cancer stem cells. Sandiford and colleagues (61) demonstrated that MSCs, primed by prior exposure to breast cancer cells, produced EOs that induced cancer cell transition to a cancer stem cell-like state. Similar to our data presented here, Sandiford and colleagues found that MSC-derived EOs altered cell cycle and promoted chemotherapy resistance in a subset of breast cancer cells (61). Our data provide further evidence of another cell type in the bone microenvironment (i.e., EOs) that may contribute to cancer progression by influencing cancer proliferation and chemotherapy response. In summary, our data indicate that EO-derived sEVs suppress breast cancer proliferation, in part through regulation of ERK1/2 signaling (Fig. 7G). We also showed that miR-148a-3p, enriched in EO sEVs, is capable of regulating breast cancer proliferation and levels of ERK2 mRNA and protein. Ultimately, sEV-mediated communication between EOs and breast cancer cells may be one mechanism by which the bone microenvironment influences cancer progression and suppresses proliferation of bone disseminated cancer cells.

Our data provide further evidence of another cell type in the bone microenvironment (i.e., EOs) that may contribute to cancer progression by influencing cancer proliferation and chemotherapy response. In summary, our data indicate that EO-derived sEVs suppress breast cancer proliferation, in part through regulation of ERK1/2 signaling (Fig. 7G). We also showed that miR-148a-3p, enriched in EO sEVs, is capable of regulating breast cancer proliferation and levels of ERK2 mRNA and protein. Ultimately, sEV-mediated communication between EOs and breast cancer cells may be one mechanism by which the bone microenvironment influences cancer progression and suppresses proliferation of bone disseminated cancer cells.

EO-Derived EVs Suppress Breast Cancer Proliferation

In this study, we identified miR-148a-3p as being abundant in EO-derived sEVs, and capable of suppressing breast cancer proliferation (Fig. 7A–C). Importantly, transfection with a miR-148a-3p inhibitor rescued proliferation when cancer cells were exposed to EO-derived sEVs (Fig. 7D), suggesting that miR-148a-3p, enriched in EO-derived sEVs, is a key factor in regulating breast cancer proliferation. Breast cancer cells transfected with miR-148a-3p mimic had increased p27, but not p21 (Supplementary Fig. S11). Using TargetScan it was found that there are no predicted miR-148a-3p–binding sites in the CDKN1A (p21) transcript, and there is one predicted miR-148a-3p–binding site in the CDKN1B (p27) transcript (39). Despite the fact that there is a potential miR-148a-3p–binding site in CDKN1B, in our study we observed that transfection with a miR-148a-3p mimic actually increased...
Shupp et al.

Authors’ Disclosures
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Authors’ Contributions
A.B. Shupp: Conceptualization, data curation, formal analysis, visualization, methodology, writing—original draft, writing—review and editing. M. Neupane: Data curation, visualization, methodology, writing—review and editing. L.C. Agostini: Data curation, visualization, methodology, writing—review and editing. G. Ning: Resources, data curation, visualization, methodology, writing—review and editing. J.R. Brody: Resources, methodology, writing—review and editing. K.M. Bussard: Conceptualization, resources, data curation, supervision, funding acquisition, investigation, methodology, writing—review and editing.

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