Extracellular vesicles derived from oxidatively stressed stromal cells promote cancer progression

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SUMMARY

Like many diseases, cancer is influenced by oxidative stress. Increases in oxidative stress caused by factors such as environmental stressors and unhealthy diets can increase the risk of cancer and worsen cancer prognoses. However, oxidative stress does not always promote cancer. On the contrary, many chemotherapeutics induce the production of oxidative stress to kill cancer cells. The tumor microenvironment (TME) is another factor in cancer progression. Stromal cells, a part of the TME, communicate with cancer cells through extracellular vesicles (EVs) - the primary vehicles for the delivery of messengers, including proteins, metabolites, DNA, and RNA. We hypothesized that the TME mediates cancer's response to oxidative stress by delivering EVs to cancer cells. In order to test this hypothesis, we treated breast cancer cells (HCC1806, SKBR3, MCF7, and MDA-MB-436) and lung cancer cells (A549 and PC9) with EVs derived from oxidatively stressed stromal cells and compared their expression of marker proteins of cell proliferation and epithelial to mesenchymal transition to cancer cells treated with EVs from control stromal cells and untreated cancer cells. We found that EVs extracted from oxidatively stressed adipocytes increased the cell proliferation of breast cancer cells. Additionally, we found that EVs extracted from lung fibroblasts promoted epithelial-mesenchymal transition in lung cancer cells. These findings present a novel way that the TME influences cancer progression. The findings of this paper can inform future cancer prevention studies.

INTRODUCTION

Cancer is a disease that primarily affects the elderly and is, thus, a significant barrier to increasing life expectancy. Breast and lung cancers are the most common, making up a quarter of all new cancer cases in the United States (1). The number of breast cancer cases has steadily risen by 0.5% per year since 2000, and there will be an estimated 236,740 new lung cancer cases in 2022 (1). Many factors, including age, smoking, obesity, unhealthy diet, etc., contribute to cancer risk. Many of these risk factors also produce oxidative stress (2). Some causes of oxidative stress include environmental stressors such as radiation and pollution, mental stressors

such as anxiety and depression, and physiological stressors such as smoking and unhealthy diets (3-6). Oxidative stress resulting from factors like pollution or smoking is associated with cancer progression, and a strong connection exists between anxiety, oxidative stress, and cancer outcomes, with worse anxiety correlating with poorer outcomes (3-6). However, oxidative stress produced by cancer therapies, paradoxically, can also promote apoptosis, or cell death, in cancer instead of being associated with cancer progression.

Most of the excess free radicals produced by the body are reactive oxygen species (ROS). This umbrella term refers to all highly reactive chemicals formed from oxygen (O_2) through redox reactions, electronic excitation, and standard processes of aerobic function (7). Researchers are eager to study oxidative stress because it is implicated in many diseases. Cancer is an example; intracellular ROS promote tumorigenesis through DNA damage and oncogene mutations (8).

The reaction of cancer cells to oxidative stress is dependent on the amount, time, and source of oxidative stress. For example, oxidative stress from stressors such as pollution, anxiety, or smoking promotes cancer progression, while oxidative stress produced by cancer therapies promotes apoptosis in cancer (2, 4-6). ROS, produced by stressors or in small doses, not only induce tumorigenesis but also activates signaling pathways to stimulate tumor proliferation and metastasis (2, 11-13). Metastasis is the development of secondary cancerous growths made up of cancerous cells that have migrated from the primary cancer. During epithelial-mesenchymal transition (EMT), epithelial cells transform morphologically into mesenchymal cells, losing epithelial characteristics and gaining mesenchymal qualities. EMT occurs during cancer progression, making cancer cells more motile and allowing cancer cells to leave the primary tumor and migrate to other tissues (10).

However, large doses of ROS damage tumor cells and cause apoptosis instead of promoting tumor growth. Many cancer therapies, including chemotherapy, specifically damage cancer cells by substantially increasing oxidative stress within them (14). Tumor cells quickly form resistance to such therapies, and the surviving cells proliferate faster than before the treatment (15). We believe that the tumor microenvironment (TME) may play a significant role in facilitating this proliferative behavior.

The TME comprises tumor cells, immune cells, non-cellular components, and tumor stromal cells such as endothelial cells and fibroblasts (16). Of particular interest is the TME's crucial role in tumor progression (17). Lung cancer fibroblasts (a primary component of the TME of most solid lung tumors), for instance, are involved in lung tissue fibrosis and inflammation, which facilitates lung cancer progression (18). Analogous to fibroblasts in lung cancer, adipocytes, abundant in the breast TME, act as critical regulators of breast tumor growth and metastasis (19). Cancer stromal cells, which facilitate cancer progression, frequently communicate with tumor cells. Extracellular vesicles (EVs) are the primary vehicles through which messengers and other biomolecules are delivered between stromal cells and tumor cells (20). In cancer, these messengers and biomolecules can cause the recipient cells to undergo phenotypic changes that promote various aspects of cancer progression (21).

We hypothesized that the EVs produced by the cancer stromal cells are responsible for the duality of the effects of oxidative stress on cancer cells, both promoting apoptosis of cancer cells in cancer therapies and causing poorer cancer outcomes. To assess this assumption, we observed the *in vitro* effect of hydrogen peroxide (H_2O_2), which is the most commonly used molecule to induce oxidative stress in cell cultures, on EVs derived from stromal cells, specifically fibroblasts, and adipocytes, and how these EVs affect lung and breast cancer cells. Adipocytes and fibroblasts were dosed with H_2O_2, EVs were isolated from the conditioned medium, cancer cells were treated with EVs, and the proliferation and EMT markers of the cancer cells were measured (**Figure 1**).

We found that 50 µM of H_2O_2 can activate lung fibroblasts and decrease the number of lipid droplets in adipocytes. Furthermore, the EVs isolated from H_2O_2-treated fibroblasts may promote EMT in lung cancer cells, and EVs isolated from H_2O_2-treated adipocytes may promote breast cancer cell proliferation. These results suggest that the EVs produced by oxidatively stressed stromal cells increase the proliferation and promote epithelial to mesenchymal transition of cancer cells. The correlation between oxidative stress, cancer cell proliferation, and metastasis may explain why cancer patients with higher stress levels (mental, physiological, or otherwise) have worse prognoses than those with less stress (21, 22).

RESULTS
Hydrogen peroxide decreases lipid droplets in adipocytes and activates lung fibroblasts

H_2O_2 is the most commonly used molecule to induce oxidative stress in cell cultures. To explore the effect of oxidative stress on adipocytes, we treated differentiated preadipocyte 3T3-L1 cells with 50 µM H_2O_2 for 4 days. Using 50 µM of H_2O_2 no obvious cell death was observed when observed under a microscope, showing that the treatment is not lethal and could potentially be representative of oxidative stress that might occur *in vivo* (23). Once the adipocytes complete differentiation, lipid droplets will form in the cells. H_2O_2-treated adipocytes were observed to have less lipid droplet accumulation than the control group, as measured by Oil O Red staining (**Figure 2A**).

In MRC5 fibroblasts, H_2O_2 treatment did not change the proliferation rate but it did induce morphological change in these cells. H_2O_2-treated fibroblasts appeared less organized and left more migratory trails (**Figure 2B**). From these morphological changes we speculated that the H_2O_2 treatment transformed the MRC5 into cancer-associated fibroblasts. To verify this hypothesis, we measured the protein level of the myofibroblast marker α-SMA, which is typically highly expressed in cancer-associated fibroblasts. α-SMA was increased after H_2O_2 treatment, thus suggesting that H_2O_2 treatment may transform MRC5 into cancer-associated fibroblasts (**Figure 2C**).

Hydrogen peroxide does not change the particle number of secreted EVs

EVs have been reported to be the primary vehicle mediating communication between tumor cells and stromal cells. Fibroblasts and adipocytes are both central components of the TME for lung tumors and breast tumors, respectively. It has been reported that fibroblasts and adipocytes can secrete EVs to regulate tumor progression. To find out whether H_2O_2 can affect EV secretion from these stromal cells and further affect tumor progression, we pretreated adipocytes and lung fibroblasts with H_2O_2 for three days, changed to fresh medium at the end of the third day of treatment, collected the conditioned medium without the H_2O_2 after 24 hours, and isolated the EVs from the conditioned medium.
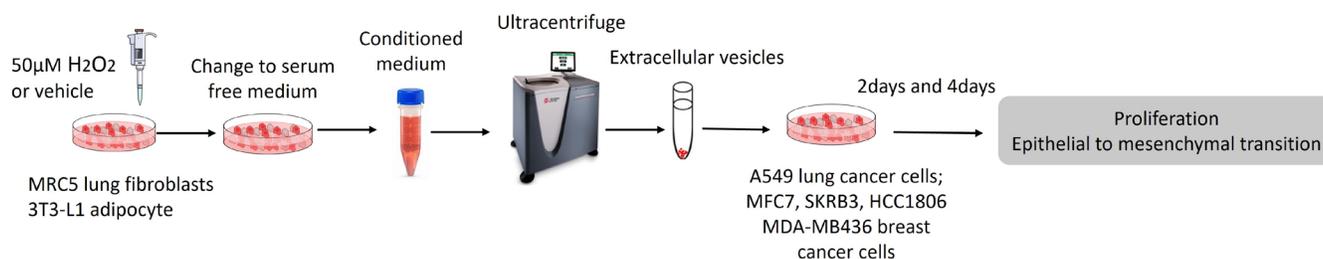

Figure 1: Outline of the experiment. Adipocytes and lung fibroblasts were treated with 50 µM H_2O_2 for 3 days, then changed to serum-free medium, and conditioned medium was collected. Extracellular vesicles (EVs) were isolated from the conditioned medium using an ultracentrifuge. A549 and PC9 lung cancer cells and MCF7, SKBR3, HCC1806, and MDA-MB-436 breast cancer cells were treated with the isolated EVs, and results were collected.
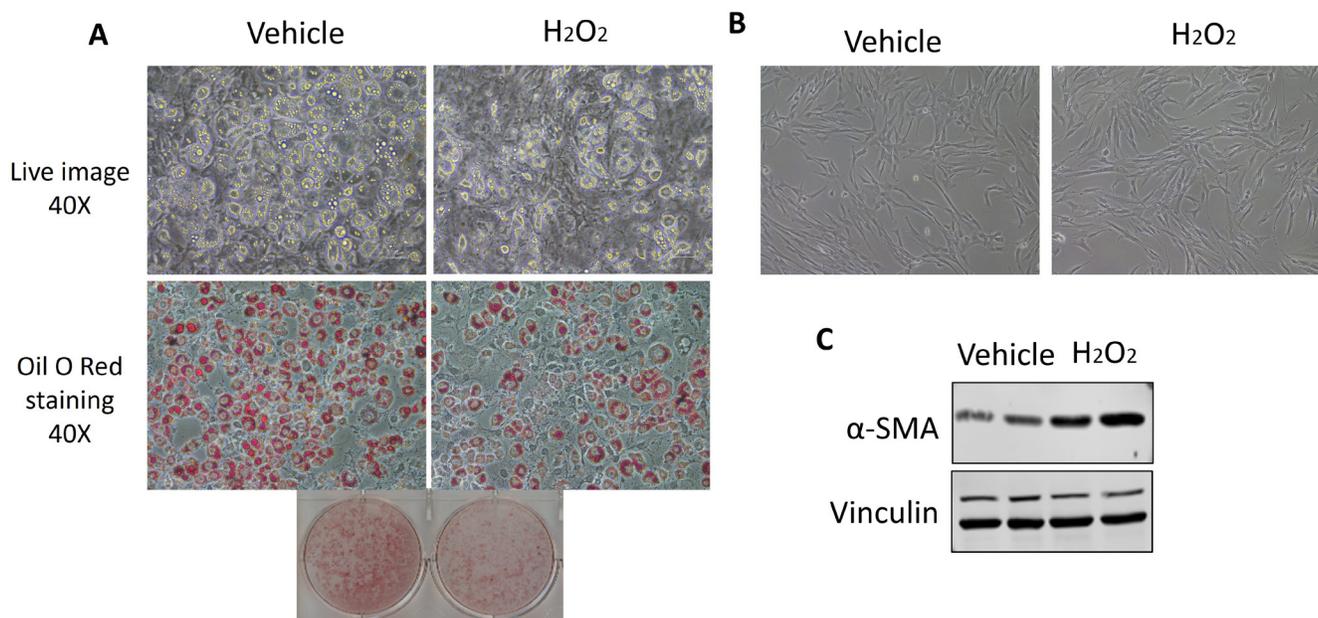
Figure 2: The effect of hydrogen peroxide on adipocytes and lung fibroblasts. A) 3T3-L1 cells were differentiated into adipocytes and treated with 50 µM H_2O_2 for 3 days. Oil O Red staining was performed to show the amount of lipid droplets in the adipocytes (red). (n = 3, images shown are representative). B) MRC5 lung fibroblasts were treated with 50µM H_2O_2 for 3 days. Images shown are representative. C) After H_2O_2 treatment, whole cell protein was extracted, and the protein level of α-SMA was measured through western blots. The two bands per treatment group are replicates.

To confirm the isolated EVs were not contaminated with cell debris, the particles in the EV solution were imaged through an electron microscope (data not shown). To determine the effect of H_2O_2 on EV secretion, we used a NanoSight instrument to count the isolated EVs, and the results showed that there was no significant difference between the amount of EVs in the control group and the H_2O_2 treatment group (**Figure 3A-B**).

Hydrogen peroxide-treated adipocyte-derived EVs promote breast cancer cell proliferation

The components of EVs can be regulated by internal or external stimuli. Since we did not see a difference in the particle numbers of EVs under H_2O_2 treatment, we wondered whether the components of EVs were changed by H_2O_2 treatment and might affect tumor progression. We added EVs extracted from H_2O_2-treated adipocytes into the medium of breast cancer cells and cultured them for 4 days. The cell number was counted to evaluate the proliferation rate. We first added EVs from 3T3-L1 cells into four breast cancer cell lines. Interestingly, we found the EVs isolated from vehicle-treated 3T3-L1 cells (Veh-EVs) did not affect the tumor cell proliferation significantly, compared to the non-treated tumor cells in all four breast cancer cell lines. However, the EVs isolated from H_2O_2-treated 3T3-L1 cells (H_2O_2-EVs) promoted proliferation in three of the four cell lines (**Figure 4A-B**). Additionally, these four breast cancer cell lines responded differently to H_2O_2-EVs. For SKBR3 cells, H_2O_2-EVs promoted proliferation quickly, as we could see a difference on day 2 after treatment (p=0.0313), and

the difference got larger on day 4 (p=0.0279). In MCF7, we did not see a significant proliferation-promoting effect on day 2 (p=0.1315), but H_2O_2-EVs significantly promoted cell proliferation on day 4 (p=0.0414). We saw a similar trend in HCC1806 cells (p=0.007) as in MCF7 cells. Surprisingly, there was no proliferative effect observed on MDA-MB-436 cells (**Figure 4A-B**).

To further understand how H_2O_2-EVs promote cell proliferation, we performed western blots to check the expression of marker proteins that regulate cell proliferation.
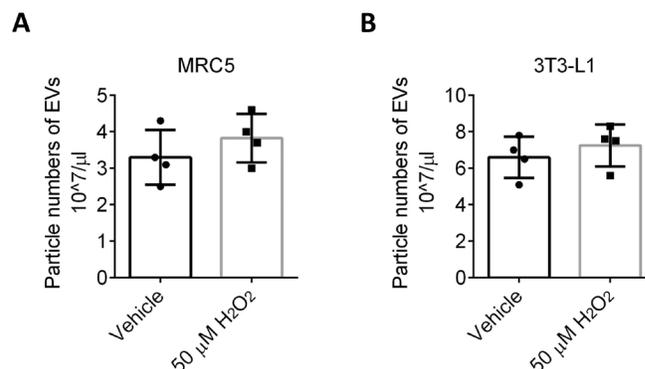

Figure 3: The effect of Hydrogen peroxide on EV secretion. Data shown as mean ± standard deviation. A) EVs were isolated from the conditioned medium of MRC5 fibroblasts. The mean particle number of EVs was counted by NanoSight. No significant difference was seen between Vehicular EVs and H_2O_2 EVs. B) EVs were isolated from the conditioned medium of 3T3-L1 adipocyte. The mean particle number of EVs was counted by NanoSight. No significant difference was seen between Vehicular EVs and H_2O_2 EVs.

First, we checked mTOR signaling, which is well-studied to regulate cell proliferation (24). The phosphorylation of S6-kinase (S6K and S6 are both indicators of activation of mTOR signaling (25). We found that H_2O_2-EVs increased the phosphorylation level of S6K and S6 in SKBR3, MCF7, and HCC1806 cells but not in MD-MBA-436 cells (**Figure 4C**). This might explain the changes we saw in cell proliferation. We also measured the phosphorylation of retinoblastoma protein (Rb), a tumor suppressor protein that impedes cell cycle progression. Rb can be inactivated by phosphorylation at the Ser 807/811 site, and the cell cycle can proceed after Rb inactivation (26). In our results, H_2O_2-EVs greatly increased the phosphorylation level of Rb in SKBR3, MCF7, and HCC1806 cells but only slightly increased Rb phosphorylation in MDA-MB-436 cells.

These results suggested that EV components were different after H_2O_2 treatment and that the changes in EVs induced by H_2O_2 can promote breast cancer cell proliferation.

Hydrogen peroxide-treated fibroblast-derived EVs promote lung cancer epithelial to mesenchymal transition

We also treated two lung cancer cell lines with EVs isolated from MRC5 lung fibroblasts after treatment with H_2O_2. We found that neither Veh-EVs nor H_2O_2-EVs changed cell proliferation significantly (**Figure 5A-B**). However, when viewed under a microscope, the cells treated with the H_2O_2-EVs looked more mesenchymal than those treated with Veh-EVs, suggesting the cells underwent EMT, a precursor to metastasis (**Figure 5A**). To confirm whether treating lung cancer cells with EVs extracted from H_2O_2-treated MRC5 promotes EMT, we investigated changes in EMT markers.
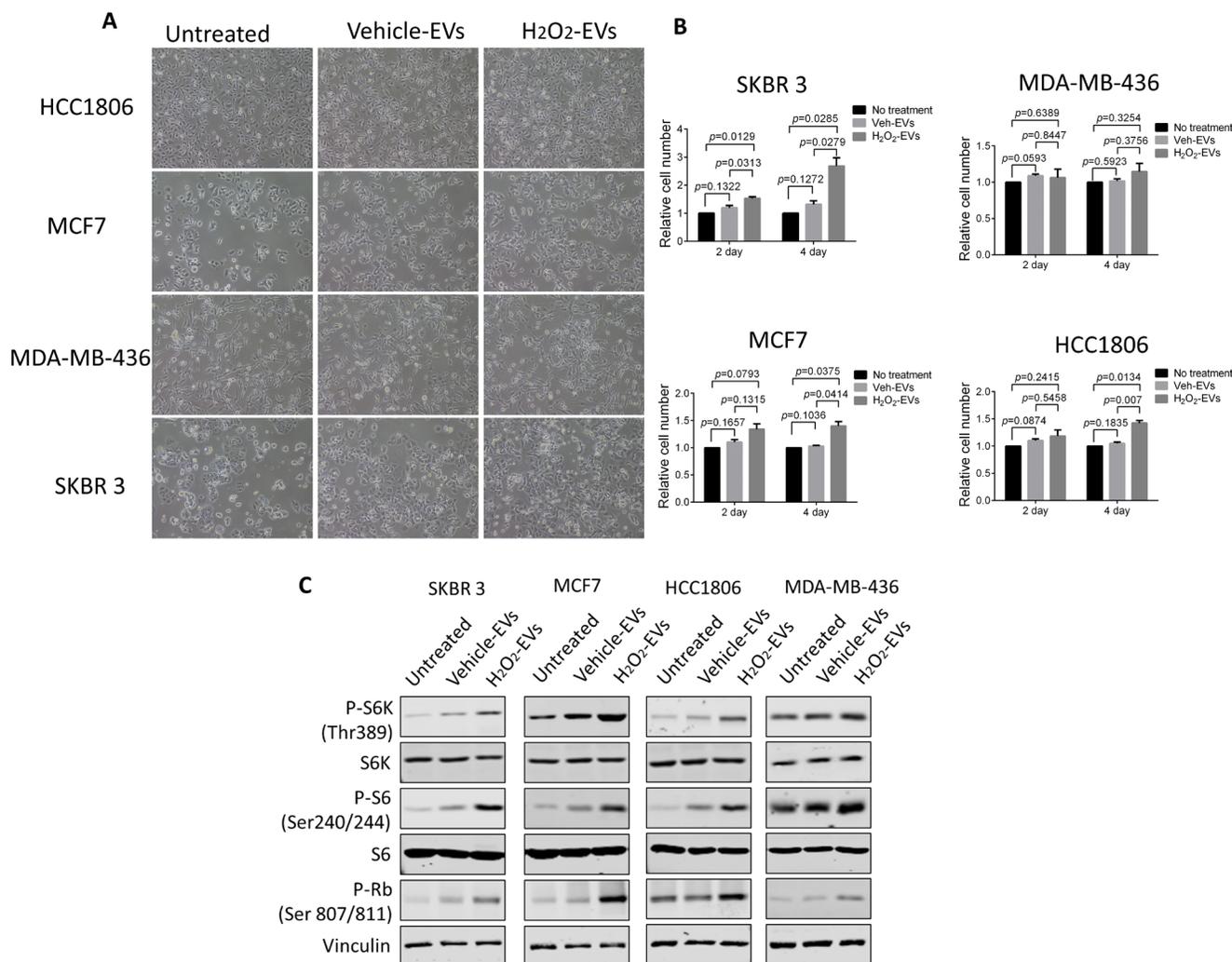

Figure 4: The effect of adipocyte-derived EVs on breast cancer cell proliferation. A) The SKBR3, MDA-MB-436, MCF7, and HCC1806 cell lines were treated with EVs isolated from control or H_2O_2-treated adipocytes for 4 days. Representative images taken at the end of the fourth day are shown. B) Mean cell number after 2-day and 4-day treatment. n=3 (number of wells counted). Error bars present standard deviation. t-test between the treatment groups (cells treated with Vehicular EVs and H_2O_2 EVs) and the no-treatment group. C) The tumor cells were treated with EVs for 4 days, and total cell protein was collected. Representative blots indicated the activity of the mTOR signaling pathway and Rb protein.

When cells undergo EMT, mesenchymal markers increase, and epithelial markers decrease. Fibronectin, vimentin, and plasminogen activator inhibitor-1 (PAI-1) are all mesenchymal markers, and E-cadherin is an epithelial marker. In both A549 and PC9 cells, treatment with H_2O_2-EVs increased the highest amounts of mesenchymal markers and decreased the amounts of E-cadherin (**Figure 5C**). Additionally, in A549, vehicle-EVs slightly increased mesenchymal markers and decreased epithelial markers compared to the control group; however, this change was not observed in the PC9 cells (**Figure 5C**).

DISCUSSION

Oxidative stress is involved in the formation and progression of many diseases, especially cancer. Many studies have shown that oxidative stress can regulate tumor metastasis, but there are some studies that have shown the opposite as well. Here, we explore this question from the view of tumor stromal cells. H_2O_2, the compound we used to model oxidative stress, was found to influence adipocytes, lung fibroblasts, and the EVs produced from them. When we treated adipocytes and lung fibroblasts with H_2O_2, we found that H_2O_2 decreased the amount of lipid droplets inside adipocytes. The observed decrease in lipid droplets could be the result of reduced lipid uptake, reduced lipid synthesis, or increased lipolysis. However, to figure out the mechanism, in the future, we still need to measure the activities and

expression of enzymes for fatty acid uptake, lipogenesis, and lipolysis. This decrease could be a mechanism of cachexia as one of its most common mechanisms is oxidative stress and many advanced cancer patients develop cachexia (27, 28). Cachexia is a metabolic syndrome related to muscle mass loss with or without fat mass loss. However, we have not researched enough to reach conclusions about this hypothesis. There was also a change in the lung fibroblasts when treated with H_2O_2, found through observation under microscope. Fibroblasts in the TME can be activated by many factors, can differentiate into various states, and exert different effects on tumor cells. Because the H_2O_2 did not affect the cells' proliferation or death but obviously changed them, we still need to measure more cancer-associated fibroblast markers to determine what type of fibroblasts were induced by H_2O_2.

An interesting occurrence is the different effects of EVs on breast and lung cancer cells. When treated with EVs extracted from H_2O_2-treated adipocytes, the breast cancer cells had increased proliferation but no change in morphology. However, the treated lung cancer cells did not show an increase in proliferation but did show signs of epithelial to mesenchymal transition. The different contents of EVs from adipocytes and fibroblasts are likely the cause of this phenomenon. The EVs from adipocytes may contain more molecules that can produce energy, like fatty acids, which can promote proliferation (29). In terms of EV components, we
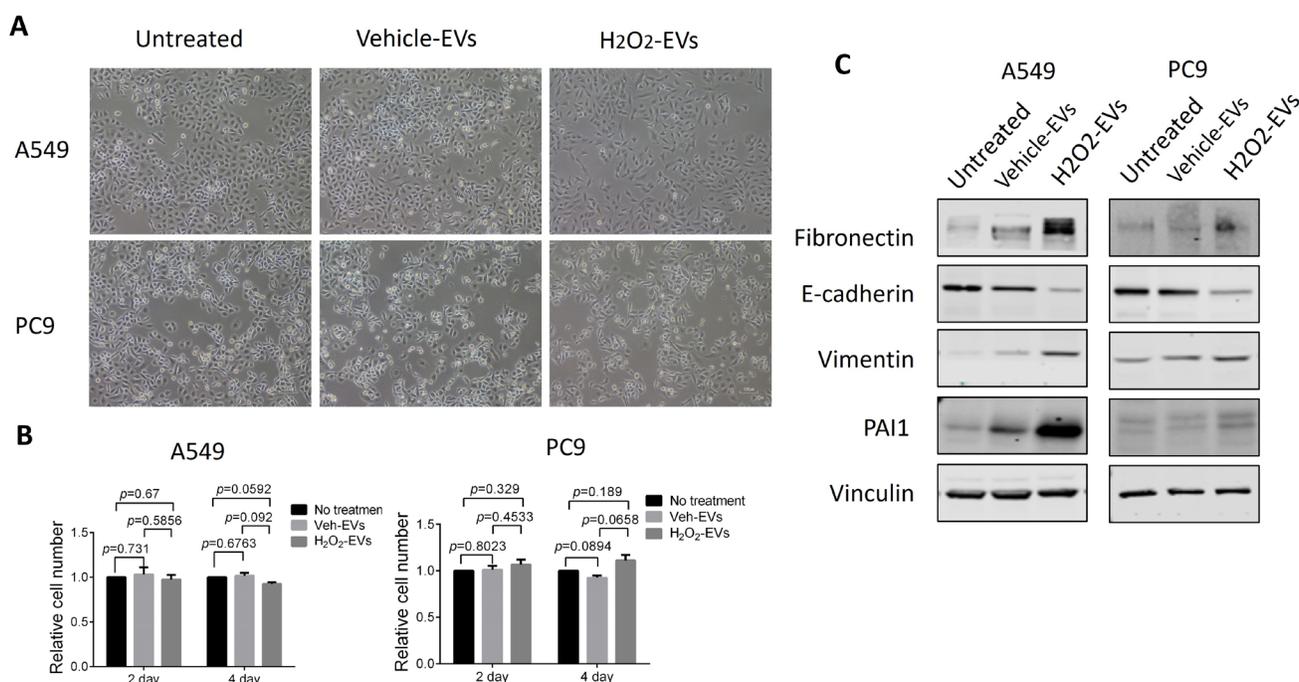

Figure 5: The effect of MRC5-derived EVs on lung cancer cell proliferation and EMT. A) A549 and PC9 were treated with EVs isolated from MRC5 fibroblasts for 4 days. Representative images taken at the end of the fourth day are shown. B) Mean number of tumor cells on day 2 and day 4. n=3 (number of wells counted). Error bars present standard deviation. No significant difference was seen between the treatment groups (Vehicular EVs and H_2O_2 EVs) and the no-treatment group for the 2-day A549 cells, for the 4-day A549 cells, for the 2-day PC9 cells, and for the 4-day PC9 cells. C) The tumor cells were treated with EVs for 4 days, and total cell protein was collected. Representative blots were shown to indicate the expression of epithelial markers and mesenchymal markers.

still need to perform proteomics and metabolomics analysis to figure out the critical factors in the EVs inducing such differences. Additionally, the measurement of the protein level of EV markers, cell nuclei, and mitochondrial markers is still necessary to confirm the purity of the isolated EVs.

Another interesting phenomenon was that the different breast cancer cell lines had varying degrees of responsiveness to the EVs regardless of whether they were extracted from cells treated with H_2O_2 or not. The difference in responsiveness could be due to differences in the aggressiveness of the breast cancer cell lines. Breast cancer cell lines are classified by the presence or absence of estrogen receptors (ER) and human epithelial receptors 2 (HER2). Breast cancer cell lines can be classified (listed from least to most aggressive) into ER positive (ER+), HER2 positive (HER+), and triple negative, which lacks the ER, HER2, and progesterone receptors (27). The MCF7 line is ER+, the SKBR3 line is HER2+, and the HCC1806 and MDA-MB-436 lines are triple negative. The two least aggressive cell lines showed increased proliferation when treated with H_2O_2-EVs after 4 days, but the MDA-MB-436 line, one of the aggressive cell lines, showed no differences in proliferation. However, HCC1806 had increased proliferation with H_2O_2-EV treatment after 4 days. This discrepancy is difficult to interpret; the classification of the cell line is not enough to predict its response to the EV treatment. Thus, more experiments with more cell lines and under different concentrations and lengths of H_2O_2-EV treatment are needed to explain the different responses to EVs.

Overall, our studies suggest that EVs secreted by tumor stromal cells under oxidative stress promote cancer progression, which offers a new mechanism for regulating oxidative stress on cancer progression. However, there are still limitations to the study. The 3T3-L1 cell line, while commonly used for studying human adipocytes and cancer, originates from mice, and this mouse origin may introduce subtle differences in the results when compared to human adipose cells. H_2O_2 is the most common form of ROS in cells and is widespread in its use in oxidative stress studies (23). However, the source and form of oxidative stress - not just the amount - affects the cell's response. To understand the effects of oxidative stress more holistically, we need to use other techniques to model oxidative stress conditions. The entirety of the research done was *in vitro*. Because cell interactions *in vivo* are much more complex, it is difficult to determine how well our conclusions and observations will hold up *in vivo*. Additionally, we made observations after only four days of treatment. It is unclear whether long-term treatment may have different effects on the cancer cells.

Oxidative stress can increase the risk of developing cancer. The increased risk may have to do with regulating the TME. According to our results, oxidative stress may promote cancer proliferation and metastasis by regulating stromal cells' production of EVs.

MATERIALS AND METHODS
Cell culture
Cell lines were obtained through the American Type Culture Collection (ATCC). The cells used were lung adenocarcinoma cells (A549 and PC9), breast cancer cells (HCC1806, SKBR3, MCF7, and MDA-MB-436), human lung fibroblasts (MRC-5), and mouse preadipocytes (3T3-L1). A549, PC9, HCC1806, and SKBR3 cells were cultured in a mixture of 10% FBS (Sigma-Aldrich), RPMI 1640 medium (Corning), and 1% penicillin-streptomycin (Gibco). MCF7, MDA-MB-436, and 3T3-L1 were cultured in a mixture of 10% FBS (Sigma-Aldrich), high-glucose DMEM (Gibco), and 1% penicillin-streptomycin (Gibco). MRC-5 cells were cultured in 10% FBS (Sigma-Aldrich), EMEM (ATCC), and 1% penicillin-streptomycin (Gibco).

The cell medium was changed every day, and the cells were maintained at 5% CO_2 and 37°C. MRC5 fibroblasts and 3T3-L1 adipocytes were treated with 50 µM H_2O_2 or H_2O (control/vehicle) for 3 days. Both breast and lung cancer cells were treated with 5 µg/mL of EVs for 2 and 4 days each. The cells were counted at the end of the second and fourth day of treatment using a Multisizer 3 Coulter Counter (Beckman Coulter). To collect the cells, 0.5 mL of trypsin (to detach cells from the plate) was added to each cell plate, followed by 5 mL of medium (to neutralize the trypsin). To determine the statistical significance of the cell counts, Google Sheets was used to calculate T-tests with alpha levels set to 0.05. Live cell images were taken by a Leica microscope.

Western blotting
Cells were lysed using RIPA buffer in the plates they were cultured in. The buffer consists of 40 mM HEPES [pH 7.4], 1 mM EDTA, 120 mM NaCl, 0.5 mM DTT, 10 mM b-glycerophosphate, 1 mM NaF, 1 mM Na3VO4, 0.1% Brij-35, 0.1% deoxycholate, and 0.5% NP-40. Protease inhibitors (250 mM PMSF, 5 mg/mL pepstatin A, 10 mg/Ll leupeptin, and 5 mg/mL aprotinin) were also added to prevent protein from breaking down. The cell lysate was then incubated at 4°C for 15 minutes, followed by incubation with 4X LDS at 90°C for 5 minutes. Protein quantification was done using the DC Protein Assay Kit II (BioRad). 30 µg of protein from each lysate was run on SDS-PAGE gels. The resultant separated proteins were transferred to a nitrocellulose membrane submerged in TBS-based Odyssey Blocking buffer (LI-COR) using electrophoresis. The antibodies and dilutions used to detect proteins of interest on the nitrocellulose membrane are as listed: α-SMA (ab5694 – Abcam, 1:1000), E-Cadherin (610181 – BD, 1:1000), PAI-1 (612024 – BD, 1:1000), Vimentin (5741S – Cell Signaling Technology, 1:2000), Vinculin (V9264 – Sigma Aldrich, 1:5000), and Fibronectin (ab2413 – Abcam, 1:10,000).

Extracellular vesicle extraction
The MRC5 and 3T3-L1 cells were washed with PBS at 1x concentration twice after removing the medium. Next, a

medium without serum was added to the cells. The conditioned medium (CM) was collected after 2 days. To extract the EVs from the collected medium, the CM was centrifuged at 2,000xg for 15 minutes, followed by 12,000 for 20 minutes. The supernatant was extracted and centrifuged at 100,000xg for 70 minutes. The supernatant was discarded, resuspended in PBS at 1x concentration, and centrifuged once more at 100,000xg for 70 minutes. Finally, protein quantification was done using DC Protein Assay Kit II (BioRad), and the number of particles was counted using Nanosight NS500. To determine statistical significance of the number of particles, Google Sheets was used to calculate T-tests with alpha levels set to 0.05 (5%).

3T3-L1 cell differentiation

3T3-L1 cells were cultured in a mixture of 10% FBS (Sigma-Aldrich), high-glucose DMEM (Gibco), and 1% penicillin-streptomycin (Gibco). After having reached a confluence greater than 80%, the adipocytes were left inside the incubator for 3 days. To induce differentiation, the medium made up of 50μM IBMX, 250nM dexamethasone, 1μM rosiglitazone, and 170nM insulin in DMEM was added to the starving adipocytes for 3 days. For the next 3 days, medium made up of 170nM insulin in DMEM was used to maintain the differentiation of the adipocytes.

Oil O Red staining

Oil O Red staining was used to stain the lipids that formed in the adipocytes. The medium was removed, and the adipocytes were washed with PBS at 1x concentration twice. Next, 4% formalin was added, and the cells were incubated for 30 minutes. The formalin was removed, and the cells were washed with PBS at 1x concentration twice. After that, 60% isopropanol was used to incubate the cells for 5 minutes. After the isopropanol was removed, Oil O Red solution (Sigma) was added to cover the cells for 15 minutes. After washing the cells with PBS at 1x concentration, the stained images were taken using a microscope at 40x magnification.

Electron microscope imaging

EVs were processed for electron microscope (EM) imaging as previously described (23). Briefly, frozen EVs were thawed and fixed on ice for 5 min in 2% PFA (EMS, 15710). Then, the sample was deposited on formvar/carbon-coated nickel grids (EMS, FCF400H-NI-SB), fixed for 5 min in 1% glutaraldehyde (EMS, 16120), contrasted for 5 min with 4% uranyl oxalate, and finally embedded in 2% methyl cellulose (Sigma, M6385) and uranyl acetate solution (EMS 22400). Images were acquired using a JEOL JEM 1400 transmission electron microscope (JEOL, USA, Inc, Peabody, MA) at 100keV equipped with a Veleta 2 K x 2K CCD (EMSIS, GmbH, Muenster, Germany).

Statistics

All measurements utilized for statistical analyses in independent experiments were obtained from different samples. Data analyses were conducted using Microsoft Excel 2013 and GraphPad Prism7. Unless explicitly stated otherwise, results are presented as mean±SEM. Significance was determined using the two-tailed Student's t-test. The p-values indicating significant differences between groups are presented in the results. In the case of western blots, experiments were independently replicated n=3 times, and representative images are depicted.

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