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EFFECTS OF EXERCISE-INDUCED EXTRACELLULAR VESICLES ON TRIPLE NEGATIVE BREAST CANCER CELLS

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THE EFFECTS OF EXERCISE-INDUCED EXTRACELLULAR VESICLES ON TRIPLE NEGATIVE BREAST CANCER CELLS

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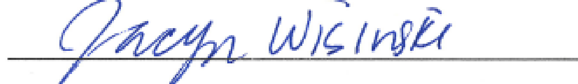

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ABSTRACT

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Triple negative breast cancer (TNBC) is an aggressive form of breast cancer that is extremely difficult to treat due to the lack of progesterone, estrogen, and Her2 receptors in and/or on the cell. Currently, indicated treatment methods include surgery, radiation therapy, and chemotherapy, which are invasive and generally have negative side effects on the patient. This creates the need for a less invasive and more effective treatment method. Ongoing research shows that exercise can decrease TNBC tumor growth, suggesting that physical activity releases a systemic mediator of cancer growth into the bloodstream. It is hypothesized that these mediators are released via a type of extracellular vesicle. Extracellular vesicles (EVs) can carry miRNAs that cause repression of protein translation, perhaps resulting in downstream effects relevant to cancer survival, proliferation, and invasiveness. These miRNAs could be identified and potentially used to target and treat TNBC tumors. In this study, healthy male participants completed acute resistance exercise training and had blood taken before and after exercise, which was used to isolate EVs. We gathered preliminary data suggesting that EV treatment reduces the proliferation of a TNBC cell line, but not of an epithelial mammary cell line. Additionally, using phospho-arrays, we observed a decrease in activation status of several signaling proteins in EV treated TNBC cells, which may be contributing to the changes in proliferation. The goal of this study was to determine the effects of exercise induced EVs on proliferation, phosphorylation of kinases, as well as markers of differentiation and apoptosis to better understand the molecular mechanisms through which exercise slows tumor growth in TNBC.

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INTRODUCTION

Prevalence and Classification of Breast Cancer

The prevalence of cancer is staggering, as nearly 2 million people were diagnosed with the disease in 2023 causing the most deaths in the United States after heart disease (Siegel *et al.* 2023; CDC, 2023). Breast cancer is the most diagnosed cancer in women, with about 264,000 females diagnosed every year (National Cancer Institute, 2023). There are a variety of breast cancers, with different characteristics and mechanisms of tumor progression, causing some to be more aggressive than others. This creates the need for a variety of treatment methods that are specific to the type of breast cancer.

Breast cancer can be classified by the presence of receptors on the cancer cells. When diagnosing breast cancer, physicians will often test for the presence and/or absence of progesterone receptors (PR), estrogen receptors (ER), and human epidermal growth factor receptors (HER2). Chemotherapeutics have been designed to specifically target these receptors so that once aggressive cancers, such as HER2-positive breast cancer, are now highly survivable (BCRF, 2023). Generally, the hormone receptor status is used to identify the best treatment method for that patient. Although, if the test comes back negative for these three receptors, the breast cancer is considered triple negative (Figure 1).

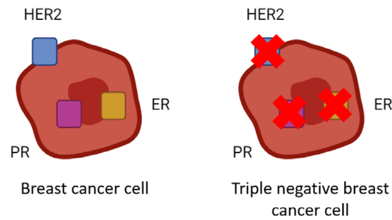

Figure 1. Presence of absence of hormone receptors in breast cancer cells.

Prevalence and Challenges of Triple Negative Breast Cancer

Triple negative breast cancer (TNBC) accounts for 12.5%-15% of all breast cancers and is more aggressive than other forms of breast cancer (Badowska-Kozakiewicz and Budzik 2016). Women diagnosed with this disease are generally premenopausal and under the age of 40 (Morris *et al.* 2007). Additionally, TNBC tumors are highly invasive, causing about 46% of patients to have distant metastasis to organs such as the brain, lungs, and liver (Lin *et al.* 2008). The 5-year survival rate for TNBC patients with regional tumors and distant metastasis is 65% and 11%, respectively (Kohler et al. 2015). The 5-year survival rate for patients with less aggressive forms of breast cancer are 86% for regional tumors and 31% for distant metastasis (National Cancer Institute, 2023).

In addition to its aggressive nature, TNBC is challenging to treat due to its lack of ER, PR, and HER2 receptors, as these receptors can serve as a target for therapeutic treatments. Cancer drugs can bind to these receptors and inhibit pathways in the cancer cell that lead to tumor progression. Due to the lack of receptors, most TNBC treatments are non-specific and invasive, including surgery, radiation therapy, and chemotherapy (ACS, 2022). Patients undergoing these therapies may experience a variety of negative short and long-term effects, including infertility, increased risk for developing other

cancers, cognitive impairments, and fatigue (Mayo Clinic, 2022; Tao *et al*. 2015). This creates a need for an effective and minimally invasive treatment for TNBC.

Beneficial Effects of Exercise on Breast Cancer Patients

Research has shown that exercise has beneficial effects on breast cancer patients, including decreased tumor growth and progression. In a study involving nearly 5,000 women with breast cancer, women who exercised regularly during the first six months after diagnosis had increased disease-free and overall survival rates compared to those who did not exercise (Chen *et al*. 2011). Additionally, breast cancer survivors who exercised had decreased risk of relapse due to increased immune function and drainage of lymph from their upper body (Kim *et al.* 2013). Another study showed that regular exercise (treadmill for 30 min/5 days/week) can decrease tumor volume and progression in mice inoculated with TNBC, compared to mice that did not exercise after they were inoculated with TNBC (Wennerberg *et al*. 2020). Additionally, moderate exercise in mice, prior to tumor inoculation, was shown to decrease tumor growth over time compared to sedentary mice (Vulczak *et al*. 2020).

Roles of "Exerkines" and Extracellular Vesicles

Although the beneficial effects of exercise on breast cancer have been established, the mechanism by which exercise decreases tumor growth and increases survival rates is unknown. However, research has identified molecules released during exercise that may be responsible for the health benefits seen from physical activity. These molecules, known as "exerkines" can range from cytokines, nucleic acids, lipids, and proteins, and work through endocrine, paracrine, and/or autocrine pathways (Safdar *et al.* 2016;

Vechetti *et al.* 2020). Exerkines can improve metabolic, neurological, and immune health, and help treat cardiovascular diseases, including diabetes and obesity, as well as cancer (Eckardt *et al.* 2014; Manole *et al.* 2018). They can be released by cells through a variety of methods, including extracellular vesicles (EVs), indicating that the contents of EVs could be responsible for the health benefits from exercise (Fruhbeis *et al.* 2015). Additionally, studies have shown that muscle cells secrete EVs during exercise, indicated by an increase in EV concentration post physical activity (Gorgens *et al.* 2015). Overall, this has led to the hypothesis that a systemic mediator of cancer growth is released into the bloodstream during exercise, via extracellular vesicles.

Extracellular vesicles (EVs) are released by cells and travel via bodily fluids such as blood, urine, and breast milk, allowing communication between cells (Hu *et al*. 2012). These vesicles are believed to have preferential interactions with recipient cells based on surface receptor proteins and adhesion molecules (Figure 2). EVs are categorized based on size and include apoptotic bodies (500-4000 nm), ectosomes/microvesicles (100-1000 nm), and exosomes (30-120 nm) (Elmore 2007; Cocucci *et al.* 2009; French *et al.* 2017). EVs carry a variety of contents, including proteins, lipids, and nucleic acids, that have a range of functions in cells. Of interest to this study is the function of microRNAs (miRNAs), a type of regulatory nucleic acid.

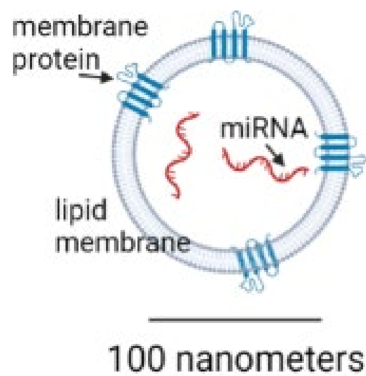

Figure 2. Extracellular vesicle containing miRNAs.

miRNAs are small non-coding RNAs that can bind to complementary mRNAs in target cells. If the miRNA is a perfect complement to the mRNA, the mRNA will be degraded, preventing it from being translated into a functional protein. If they are only a partial complement, the miRNA will temporarily repress translation of the mRNA. Studies estimate that miRNAs regulate about 1/3 of the mammalian genome, suggesting that they play a significant role in regulating gene expression (Romao *et al.* 2014). Altering gene expression can modify cell signaling and affect the cells' ability to carry out functions such as proliferation, differentiation, and migration (Hu *et al.* 2012).

A recent study examined the miRNAs released via extracellular vesicles during exercise and their effect on breast cancer. miR-206 was upregulated in EVs released during exercise and is an indicator of breast cancer prognosis and patient survival. Additionally, this miRNA can affect cell proliferation and inhibit cancer invasion. miR-30c was downregulated in exercise-induced EVs and can also affect cell proliferation. miR-204 was downregulated and plays a role in the epithelial to mesenchymal transition (EMT) and apoptosis (Pulliero *et al.* 2020). Identifying miRNAs in the EVs released during exercise can give insight into the pathways being affected in breast cancer cells.

Preliminary Data on EV Treated TNBC Cells

We have collected preliminary data suggesting that treating a TNBC cell line with EVs collected pre- and post-exercise decreases proliferation. To better understand the mechanism affecting the change in proliferation, gene expression of differentiation markers and protein levels of apoptotic markers were examined. Additionally, activation status of signaling proteins were examined to determine what cellular pathways were being affected by EV treatment.

Investigating Effects of Exercise-Induced EVs on TNBC Cells

Epithelial cells differentiate into mesenchymal cells and vice versa during development, embryogenesis, and wound healing (Hugo *et al.* 2007). This process is called the epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET). Cells gain motility when in the mesenchymal state, allowing them to move throughout the body. Once they have reached a specific location (ex- wound), the cell can transition back to the epithelial state, losing its motility. Although this is crucial for certain human processes like wound healing, cancer cells can use the EMT as a mechanism to invade other parts of the body and metastasize. Additionally, the transition to a mesenchymal state causes resistance to apoptosis, making it easier for the cancer cell to survive (Kalluri and Neilson, 2003).

The EMT can be distinguished by up and down regulation of various genes. Epithelial cells have increased expression of E-cadherin, a protein that binds epithelial cells together via adherens junctions, preventing their motility. Mesenchymal cells have increased expression of Snail, a gene induced during EMT that represses E-cadherin

expression. This action causes increased expression of N-cadherin, a mesenchymal marker that promotes cell motility. Breast cancer cells generally have increased expression of Snail, which is associated with poor prognosis, tumor recurrence, and metastasis (Barrallo-Gimeno and Nieto, 2005). Additionally, TNBC cells have higher expression of N-cadherin than mammary epithelial cells, causing them to have stem cell-like characteristics and promote tumorigenesis (Thiery 2002; Hugo *et al.* 2007; Nieman *et al.* 1999). Expression of these genes can be measured to determine if cells are differentiating to an epithelial or mesenchymal state due to EV treatment (Figure 3).

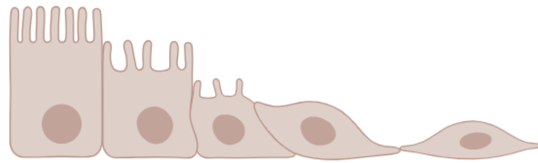

Figure 3. Characteristics and genetic markers of epithelial and mesenchymal cells undergoing EMT/MET.

Apoptosis, or programmed cell death, is a necessary process for maintenance and development of organisms (Duan *et al.* 2003). A variety of stimuli can induce apoptosis, including DNA damage or a toxic environment. This causes intracellular responses including a caspase cascade, in which caspase proteins are cleaved into an active form. Cleaved caspase-3 activates many downstream proteins involved in apoptosis, including pro-caspases 2, 6, 7, and 9, indicating its central involvement in programmed cell death (Nicholson and Thornberry, 1997). Poly ADP-ribose polymerase (PARP) is a nuclear protein that is also cleaved by caspases, serving as another indicator of apoptosis

(Kaufmann *et al.* 1993). This enzyme's main role is DNA repair, and if there is excessive DNA damage, PARP will be cleaved into an inactive state, inducing cell death (Tewari *et al.* 1995). Protein levels of cleaved caspase-3 and PARP can be measured as indicators of apoptotic pathways.

The basis of this project comes from preliminary data collected by Dr. Jennifer Klein, examining the effects of exercise-induced EVs on myoblasts. Myoblasts, or muscle precursor cells, share characteristics with TNBC cells including their stem cell-like properties (Fultang *et al.* 2021; Yu *et al.* 2021). When treated with pre-exercise and 24 hours post-exercise EVs, the myoblasts had similar levels of proliferation. Although, there was an increase in proliferation when the myoblasts were treated with EVs taken immediately after exercise. Based on this information, we wanted to explore the effects of exercise-induced EVs on TNBC cells.

This study investigates the effects of EVs isolated before and after acute resistance exercise training of male participants on proliferation of a TNBC cell line. Additionally, the pathways affecting the change in proliferation were explored, including apoptosis, differentiation via EMT, intracellular signaling, and migration. Our overall goal was to better understand the mechanisms that cause EVs and their exerkine contents to decrease proliferation of TNBC cells, with the hope of supporting future research that determines a more specific and less invasive treatment method for TNBC.

MATERIALS AND METHODS

Exercise and Blood Collection

Healthy male subjects, ranging in age from 20-35 years of age, were recruited for this study. Participants underwent acute resistance exercise test (ARET) in the UW La Crosse Human Performance Lab, which consisted of six sets of ten back squat reps at 55% and 75% of their one repetition maximum, with a two-minute rest in between each set. A warmup was done by pedaling on a cycle ergometer at 60 RPM for 5 minutes, which was followed by a series of total body stretches. Blood was taken from the subjects before, immediately (IM) after, and 24 hours after exercise. Plasma was isolated from blood via centrifugation and stored at -80°C. Participants were assigned a number and their samples were labeled with the corresponding number to deidentify them. This research has received IRB approval.

Cell Lines and Tissue Culture

Cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). BT549 was used as a TNBC cell line and MCF10a as a mammary epithelial cell line. BT549 cells were cultured in RPMI with L-Glutamine (Corning Inc., Corning, NY, USA), 10% fetal bovine serum (FBS) 10 ug/mL insulin, and 1% penicillin and streptomycin. MCF10a cells were cultured in DMEM (Corning), 5% horse serum, 20 ng/mL EGF, 0.5 ug/mL hydrocortisone, 100 ng/mL cholera toxin, 10 ug/mL insulin, and 1% penicillin and streptomycin. Cells were passed two times per week and incubated at 37°C and 5% CO_2.

Proliferation Assays

BT549 and MCF10a cells were plated on a 96 well plate at a density of 20,000 cells/mL and were incubated for 24 hours. Extracellular vesicles (EVs) from all three time points were isolated using the miRCURY Exosome Serum/Plasma Kit (Qiagen, Germantown, MD, USA) from 600 uL of plasma. The EVs were then resuspended in 540 uL growth media and used to treat the cells in two different volumes (100 uL and 50 uL). Each well originally contained 100 uL of media, so to treat the cells, either 100 uL or 50 uL of the media was removed and replaced with 100 uL or 50 uL of EVs resuspended in growth media. The treated cells were incubated for 72 hours. Following treatment, the growth media was removed from the wells and cells were washed with 1X PBS two times. Cells were then fixed with ice cold methanol and incubated on ice for 10 min. Cells were stained with 0.25% crystal violet for 10 min and left to air dry for 48-72 hours. To quantify results, the crystal violet was solubilized in 30 uL of 10% acetic acid and absorbance values were measured using the Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA) at an absorbance of 590 nm. Untreated absorbances were compared to treated absorbances to determine the change in proliferation. This procedure was replicated with SUM149-PT, MDA-MB-436, and MDA-MB-231 cell lines. Unpaired *t* tests were used for all experiments to examine statistical significance of data.

RT-qPCR

In a six well plate, BT549 cells were plated and grown for 24 hours. Cells were treated with EVs from the 24-hour post exercise time point and incubated for 72 hours. To treat one well, three vials of plasma (600 uL each) were used to isolate EVs and were resuspended in 1.5 mL of growth media. To remain consistent with the 50% treatment,

half of the media was removed from each well (1.5 mL) and replaced with 1.5 mL of EVs resuspended in growth media. Treated cells were 90% confluent and untreated were 100% confluent when collected using trypsin. Cell were then spun down at 3000 rpm for 3 minutes, washed with 1X PBS and spun again. Cell pellets were stored at -20°C. RNA extraction was completed using the RNeasy Plus Mini Kit and the QIAshredder (Qiagen), following the kits procedure. RNA was stored at -80°C. The iScript cDNA synthesis kit was used to produce cDNA from 1 ug of RNA. Samples were placed in the T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) to synthesize the cDNA with the following procedure: 1) 25°C for 5 minutes, 2) 42°C for 30 minutes, 3) 85°C for 5 minutes, 4) hold at 10°C. cDNA was stored at -20°C. To complete PCR, the TaqMan Universal PCR Master Mix (Applied Biosciences, Foster City, CA, USA) was used with a variety of primers including: E-cadherin: Hs01023894_m1, N-cadherin: Hs00983056_m1, Snai1: Hs00195591_m1, and beta actin: Hs01060665_g1 (Applied Biosystems). RT-qPCR was completed using the CFX Connect Real-Time System (BioRad) using the following procedure: 1) 50°C for 2 minutes, 2) 95°C for 10 minutes, 3) 95°C for 15 seconds, 4) 60°C for 1 minute, 5) repeat steps three and four 40 times, 6) hold at 10°C. Fold change of genes was calculated using the 2-ΔΔCt method relative to B-actin levels (Zhang *et al.* 2017).

Phospho-arrays

BT549s were seeded on 10 cm tissue culture plates 24 hours prior to experimentation. IM post exercise EVs were isolated using the miRCURY Exosomes Serum/Plasma Kit (Qiagen) and used to treat cells. Ten vials of plasma (600 uL each) were used to isolate EVs and each vial was resuspended in 540 uL of growth media. 5 mL

of media was removed from the 10 cm plate and replaced with 5 mL of EVs resuspended in media. Treated cells were 80% confluent and untreated were 100% confluent after 72 hours. Lysates were collected according to the Human Phospho-RTK Array (R&D Systems, Minneapolis, MN, USA) procedure in 300 uL of lysis buffer. Lysate concentrations were compared to a BSA standard to ensure that there were equal concentrations of untreated and treated lysate. The RTK-array was conducted according to the Human Phospho-RTK Array procedure (R&D Systems). To dilute lysates, 300 uL of lysate was added to 1.2 mL of array buffer 1 and added onto blot to incubate overnight at 4°C. Anti-phospho-tyrosine HRP detection antibody was diluted 1:5,000. The phospho-array was conducted according to the Human Phospho-Kinase Antibody Array (R&D Systems). 196 uL of untreated lysates were added to 1.8 mL of array buffer 1 and 183 uL of treated lysates were added to 13 uL of lysis buffer and 1.8 mL of array buffer 1. Blots were incubated overnight at 4°C. Streptavidin-HRP was diluted 1:2,000. The Bio-Rad ChemiDoc MP Imaging System with ImageJ Software was used for imaging and quantification of the array. The percentage change between EV treated and untreated samples was calculated using this equation: ((avg volume of untreated – average volume of treated)/avg volume of untreated).

Western Blot

BT549s were seeded on 10 cm tissue culture plates 24 hours prior to experimentation. IM post exercise EVs were isolated using the miRCURY Exosomes Serum/Plasma Kit (Qiagen) and used to treat cells. EV treatment followed the same procedure as mentioned above for the phospho-arrays. After the cells incubated for 72 hours, the treated cells were 90% confluent and untreated were 100% confluent. Cells

were lysed using 400 uL of Pierce RIPA buffer (Thermo Scientific) with 0.4 uL of phosphatase and 40 uL of protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA), centrifuged, and supernatant containing the protein was collected. Supernatant was mixed with 2X sample buffer (20% SDS, 0.5M Tris, 10% glycerol, 0.5M EDTA, 0.1 mg bromophenol blue, 0.3 mL H20, 1M DTT, 1 mL BME) and then loaded into Mini-PROTEAN TGX Gels (Bio-Rad) to be separated via SDS-PAGE. Isolated proteins were transferred from the gel to nitrocellulose paper and blocked in 5% bovine serum albumin (BSA) in 1X PBS. Primary antibodies for pGSK-3a/B, PARP, caspase-3, pEGFR, pAXL, and pERK (Cell Signaling Technology, Danvers, MA, USA) were diluted 1:1,000 in 1X PBST. The primary antibody for GAPDH (Cell Signaling Technology) was diluted 1:2,000. Membranes were incubated with the primary antibody at 4°C overnight. The membranes were washed with 1X PBST and incubated with Anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology) diluted 1:10,000 in 1X PBST for two hours. Membranes were washed again with 1X PBST and incubated with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) according to manufacturer's recommendations. ChemiDOC MP (Bio-Rad) was used to image the blot. Protein levels were quantified using ImageJ and standardized to the GAPDH loading control.

Migration Assay

BT549 cells were plated in an 8.0µm pore cell culture insert (BD Biosciences, Franklin Lakes, NJ, USA) in media without FBS and inserted into a well of a 24-well plate. Each well contained 800uL of 10% FBS media. BT549 cells were plated at a density of 250,000 cells/mL. EVs were isolated from plasma from the IM post exercise

time point using the miRCURY Exosome Serum/Plasma Kit (Qiagen) and were resuspended in 100 uL of non-growth media. The EVs in non-growth media (100 uL) and 100 uL of plain non-growth media were added to the transwell insert. After 24 hours, cells were rinsed with 1X PBS and non-migratory cells were scraped from the insert membrane. Migratory cells on the bottom membrane were fixed with 100 uL of ice-cold methanol for 10 minutes and stained with 100 uL of 0.25% crystal violet for 10 minutes, for visualization. Cells in nine fields of view were counted for each insert to quantify migration.

RESULTS

Extracellular Vesicles Caused Decrease in Proliferation of BT549 Cell Line

Given the known ability of exercise to decrease tumor growth and progression, we examined the effects of extracellular vesicles (EVs) taken before and after exercise on TNBC cells. To examine this, we isolated EVs before, immediately after, and 24 hours after exercise, treated a TNBC cell line (BT549) and mammary epithelial cell line (MCF10a), and analyzed proliferation after three days. When treated with EVs resuspended in 50 uL of media, BT549 cells showed around a 50% decrease in proliferation compared to the untreated cells (no rx) at all three time points (Figure 4). An unpaired *t* test showed that $p \leq 0.05$ for all time points, indicating that the data was statistically significant.

Surprisingly, EVs taken before exercise caused as much of a decrease in proliferation as EVs isolated post-exercise. MCF10a cells had no significant change in proliferation compared to the untreated cells. When treated with 100 uL of EVs resuspended in media, both BT549 and MCF10a cells showed minimal changes in proliferation (Figure 13; Appendix A). This data suggests that EVs isolated both before and after exercise, from healthy participants, cause a decrease in proliferation of BT549 cells but not the control, MCF10a cells, which is a non-tumorigenic mammary epithelial cell line. Additionally, the decrease in proliferation was only seen when cells were treated with 50 uL of EVs.

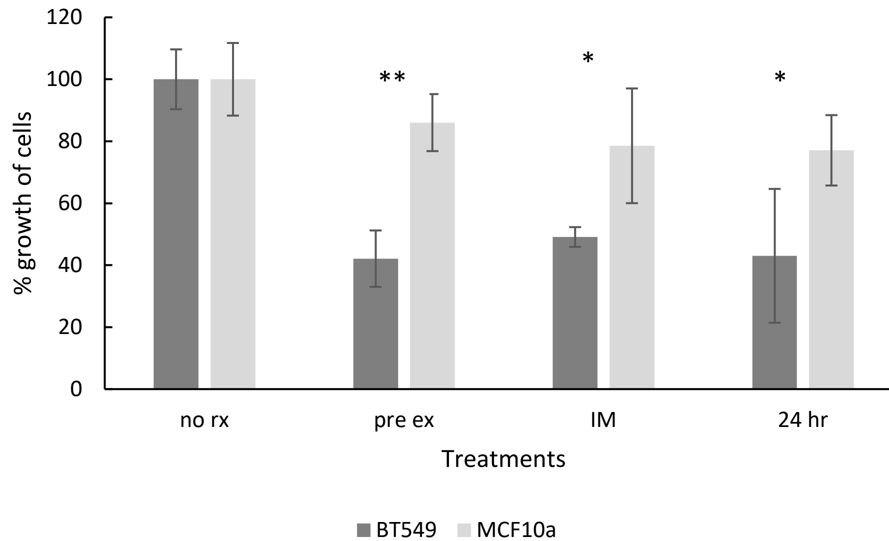

Figure 4. Percent cell growth of BT549 and MCF10a cell lines when treated with 50 uL of EVs from three time points (pre-exercise, IM post, and 24 hr post exercise), in comparison to untreated cells (no rx). Based on averages from proliferation assays using one participants EVs (n=3), including standard deviation. *=p≤0.05, *t* test. **=p≤0.005, *t* test.

TNBC cell lines are derived from different patients and tumors, causing them to have different characteristics. To determine if other TNBC cell lines saw similar effects on cell growth when treated with EVs, proliferation assays with SUM149-PT, MDA-MB-436, and MDA-MB-231 cell lines were completed. Additionally, MCF12a was used as the mammary epithelial control. When SUM149-PT cells were treated with EVs from before and after exercise, there was no significant change in proliferation at either volume (50uL or 100uL) or any time points (Figure 5) (Figure 14; Appendix A). MDA-MB-436 and MDA-MB-231 cell lines also showed no significant change in proliferation when treated with EVs at any time point or volume (Figures 15-18; Appendix A). MCF12a cells showed no significant change in proliferation when treated with EVs from all three time points, consistent with results from the other mammary epithelial cell line, MCF10a.

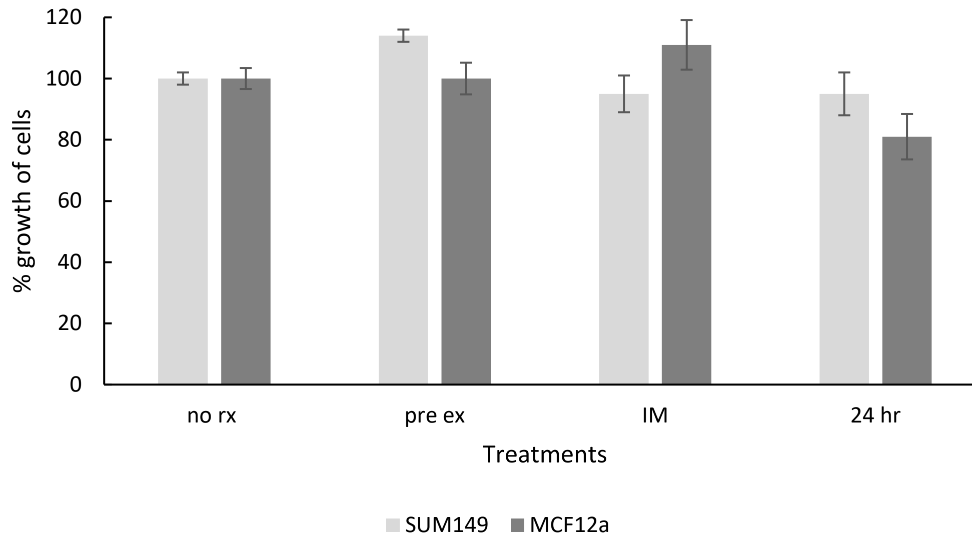

Figure 5. Percent cell growth of SUM149-PT and MCF12a cell lines when treated with 50 uL of EVs from three time points (pre-exercise, IM post, and 24 hr post exercise), in comparison to untreated cells (no rx). Based on averages from proliferation assays (n=2), including standard deviation.

Decreased Phosphorylation of Signaling Proteins in EV Treated BT549 Cells

Given the change in proliferation of EV treated metastatic cells, we wanted to determine what intracellular signaling pathways were affected. Based on the decrease in proliferation, we expected a decrease in phosphorylation of kinases affecting proliferative pathways in EV treated cells. Because BT549 cells were the only cell line that had a change in proliferation, and there were similar decreases at all three time points, we used the BT549s and the IM post-exercise EVs for future experiments. To determine which signaling pathways were altered, phospho-arrays with receptor tyrosine kinases (RTKs) and intracellular kinases were completed. RTKs are activated at the cell membrane and transmit signals intracellularly, through other kinases, causing a variety of downstream effects including changes in proliferation, differentiation, and migration. Antibodies for each kinase were spotted on the array in duplicate. Pan phospho-tyrosine antibodies were

used to identify phosphorylation of RTKs and anti phospho-tyrosine (specific to tyrosine residues on each kinase) antibodies were used to examine phosphorylation of intracellular kinases.

The phospho-RTK array showed that there was an increase in phosphorylation of EGFR, ROR, Insulin R, DDR2, Insulin R (ALK), and EphR (EphB3) in BT549 cells after treatment of IM post-exercise EVs when compared to untreated cells. There was a decrease in phosphorylation of Axl and EphR (EphA2) (Figure 6). The most observable changes were a 39.6% increase in phosphorylation of Insulin R and a 31.9% decrease in phosphorylation of EphR (Table 1; Appendix C). None of these changes were significant enough to show promise as an affected signaling pathway. Axl (21% decrease) and EGFR (26% increase) are known to function in the progression of TNBC, therefore we wanted to confirm the changes in phosphorylation observed in the phospho-array with Western blots.

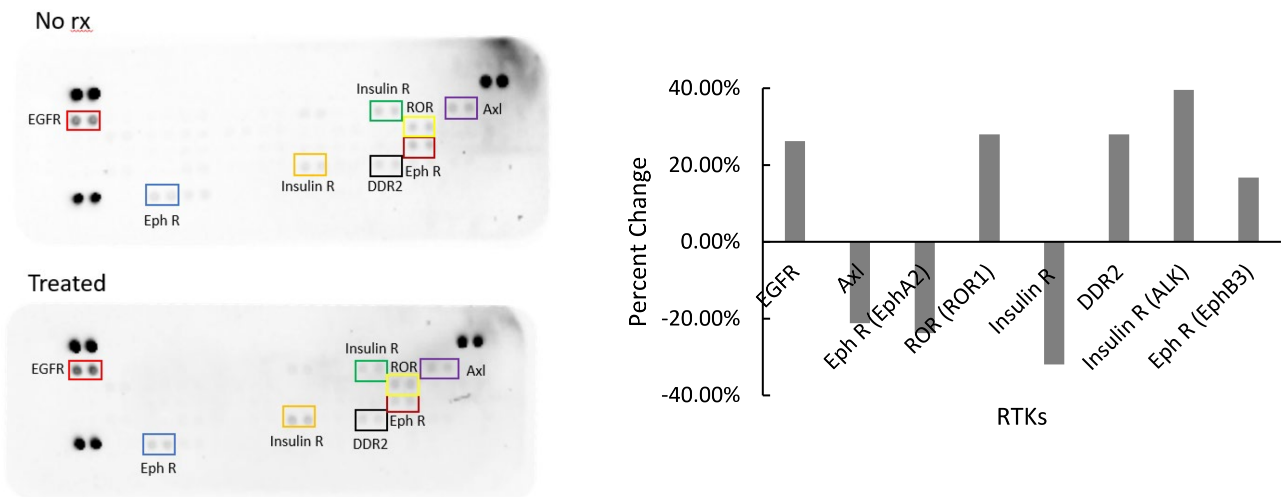

Figure 6. Phospho-RTK array comparing BT549 cells treated with IM post-exercise EVs to untreated cells. RTKs with changes in phosphorylation are labeled with colored boxes. Percent change was calculated from average integrated density.

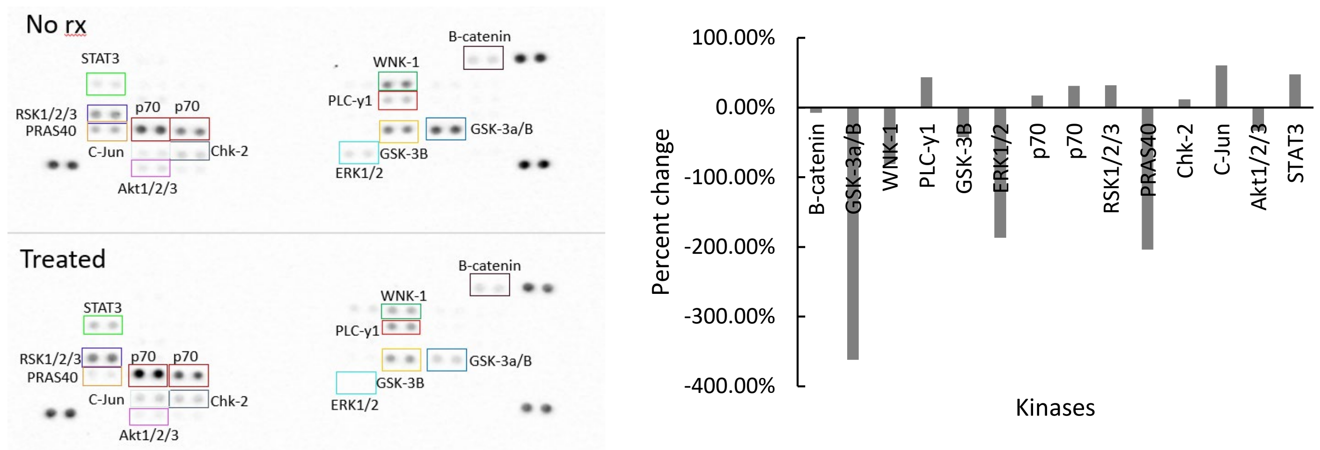

Figure 7. Phospho-kinase array comparing BT549 cells treated with IM post-exercise EV to untreated cells. Kinases with changes in phosphorylation are labeled with colored boxes. Percent change was calculated from average integrated density.

The phospho-kinase array showed that there was an increase in phosphorylation of PLC-y1, p70, RSK1/2/3, Chk-2, c-Jun, and STAT3. There was a decrease in phosphorylation of B-catenin, GSK-3a/B, WNK-1, GSK-3B, ERK1/2, PRAS40, and Akt1/2/3 (Figure 7). The most significant changes in phosphorylation were the 362% decrease of GSK-3a/B, the 187% decrease of ERK1/2, and the 204% decrease of PRAS40 when cells were treated with IM post exercise EVs, compared to the untreated cells (Table 2; Appendix C). We wanted to confirm the decreases in phosphorylation seen in GSK-3a/B and ERK1/2 with a Western blot because these kinases are known to participate in proliferative pathways.

Results from the phospho-arrays served as a preliminary search for changes in activation status of signaling proteins in EV-treated cells. This gave an idea of which cellular pathways were being affected and gave a direction for future experiments. Additionally, the kinases chosen for further examination are known to play roles in cancer pathways. Antibodies for pGSK-3a/B (ser21/9), pERK1/2 (thr202/tyr204), pAxl

(Tyr702), and pEGFR (Tyr1068) were used for Western blots to confirm the phospho-arrays. These kinases are involved in a variety of cellular functions including proliferation, migration, and EMT/MET.

Lysates were collected from EV treated and untreated cells to complete blots examining changes in phosphorylation of pGSK3a/B, pERK, pEGFR, and pAXL. Bands were not present for blots showing pERK, pEGFR, and pAxl (Figure 21; Appendix D). pGSK3a/B was able to be visualized but did not show a notable change in phosphorylation between EV treated and untreated cells (Figure 8). There were bands present at 51 kDa and 46 kDa representing the alpha and beta subunits, respectively. GAPDH served as a loading control.

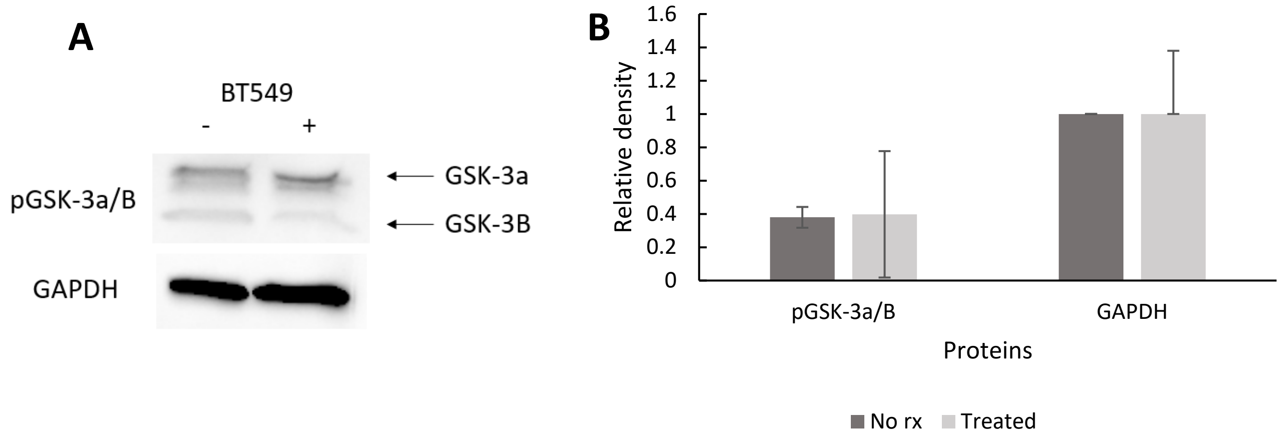

Figure 8. (A) Western blot analysis examining the presence of pGSK-3a/B in BT549 cells treated with exercise-induced EVs (IM post exercise) or untreated (no rx). GAPDH served as the loading control. (B) Quantification of Western blot, showing relative density of bands with SD (n=3).

Examination of Apoptotic Indicators PARP and Cleaved Caspase-3

The observed decrease in proliferation of EV treated cells may be due to an increase in apoptosis, therefore apoptotic indicator proteins were examined via Western

blots to determine if EVs induced cell death pathways. PARP, a DNA repair protein, and cleaved caspase-3 are common proteins involved in apoptotic pathways. The cleavage of PARP and caspase-3 would be indicators that apoptosis is occurring. Cleaved caspase-3 was not able to be visualized on the blots (Figure 22; Appendix E). PARP was present in treated and untreated cells, but cleavage of the protein, shown by the production of bands at 89 kDa and 24 kDa, was not present (Figure 9). This indicates that there was no change in the function of PARP, suggesting that induction of an apoptotic pathway did not occur.

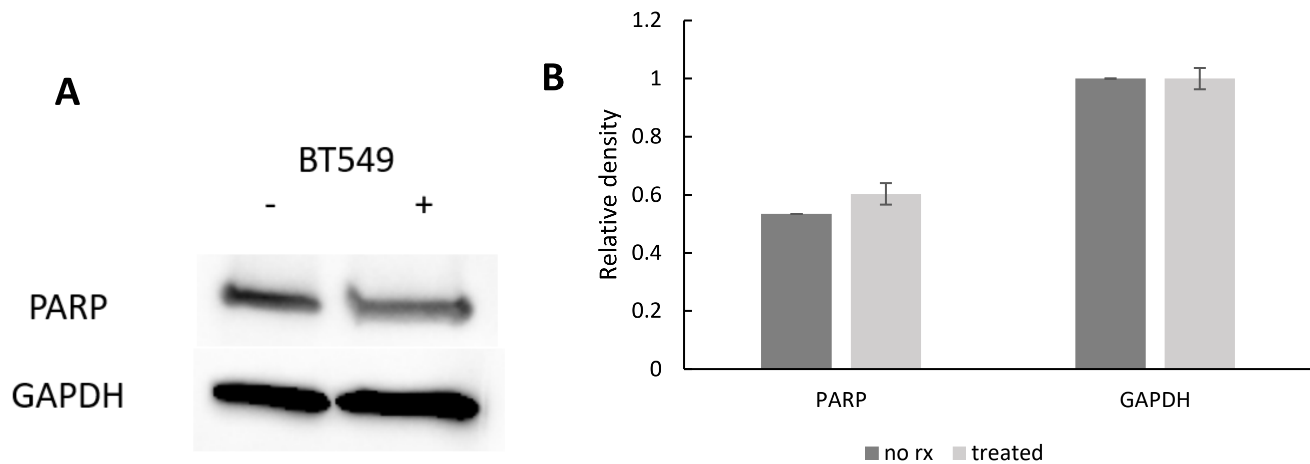

Figure 9. (A) Western blot analysis examining the presence of PARP in BT549 cells treated with exercise induced EVs (IM post exercise) or untreated (no rx). GAPDH served as the loading control. (B) Quantification of Western blot, showing relative density of bands with SD (n=3).

Gene Expression Changes of Epithelial and Mesenchymal Markers in EV Treated Cells

Due to the decrease in proliferation seen in EV treated BT549 cells, we wanted to determine if differentiation (EMT/MET) was the cause, as cells that are differentiating are less proliferative. Gene expression of epithelial and mesenchymal markers was

examined to determine if BT549 cells were undergoing an EMT or MET after being treated with 24 hr post-exercise EVs, compared to untreated cells. N-cadherin (*CDH2*) and *Snai1* served as the mesenchymal markers and E-cadherin (*CDH1*) was the epithelial marker. There was a 16-fold increase in gene expression of N-cadherin (*CDH2*), when BT549 cells were treated with 24 hr post exercise EVs (Figure 10). E-cadherin (*CDH1*) and *Snai1* had minimal changes in gene expression, with a 1.5-fold increase and a 0.66-fold decrease, respectively. An unpaired *t* test showed that p≤0.005 for the fold change in N-cadherin, indicating statistical significance. 24 hour post-exercise EVs were used in this experiment because they showed similar proliferative changes as IM post-exercise EVs.

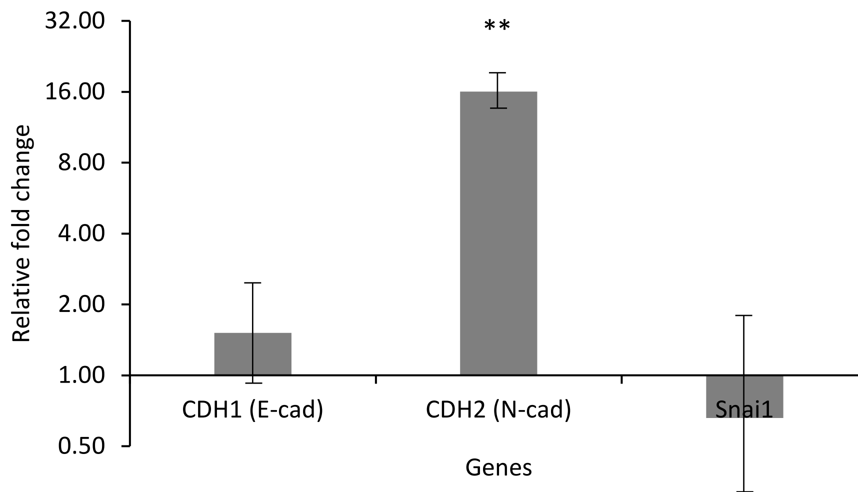

Figure 10. Expression of CDH1, CDH2, and Snai1 (SD) in BT549 cells treated with exercise-induced EVs (24 hr post exercise) compared to untreated cells. Analyzed using RT-qPCR and the ddCt method. B-actin served as the reference gene. **=p≤0.005, t test.

Migratory Effects of Exercise-Induced EVs on BT549 Cells

Due to the highly migratory characteristics of TNBC cells, we wanted to explore the impacts of exercise-induced EVs on other biological characteristics of these cells. To

do so, a functional assay assessing migration of BT549 cells treated with IM post-exercise EVs was completed. Both BT549 cells treated with IM post-exercise EVs and untreated cells had between 300-350 migratory cells after 24 hours (Figure 11). On average, BT549 cells treated with IM post-exercise EVs had 301 cells migrate through the transwell insert in 24 hours, and untreated cells had 347 cells migrate. Under the conditions we tested, migratory ability of BT549 cells were not significantly affected by the treatment of EVs.

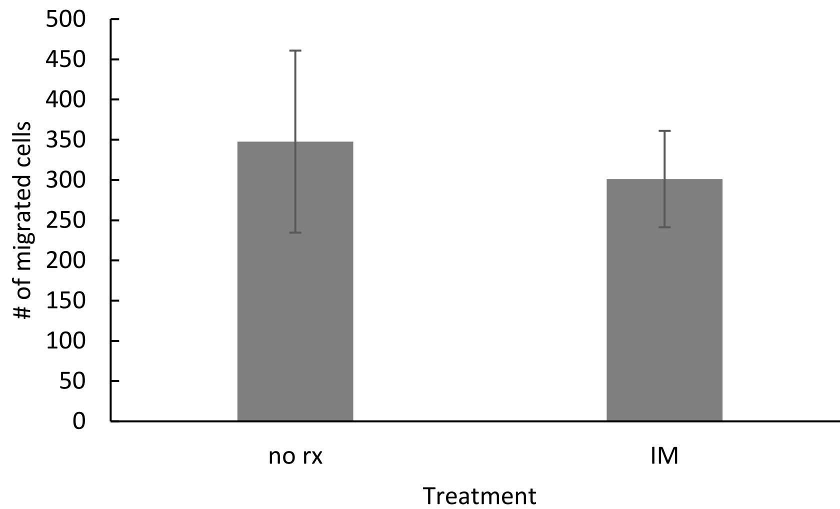

Figure 11. Relative migration of BT549 cells treated with IM post-exercise EVs and untreated, with SD.

DISCUSSION

Triple negative breast cancer (TNBC) is classified by the absence of progesterone receptors, estrogen receptors, and HER2. The disease is generally more aggressive than other types of breast cancer and challenging to treat due to its lack of previously mentioned receptors. This creates the need for a more targeted treatment method. Exercise is known to have beneficial effects on TNBC patients, including increased survival rates and decreased tumor growth, but the mechanism for these effects is unknown. This study examined the effects of exercise-induced extracellular vesicles (EVs) on molecular mechanisms in a TNBC cell line, including proliferation, gene expression of epithelial and mesenchymal markers, phosphorylation of signaling pathway intermediates, and migration.

Proliferative Changes in Exercise-Induced EV Treated TNBC Cells

Proliferation assays were initially completed to determine if there were any effects on cell division when a TNBC cell line was treated with EVs isolated before and after exercise. EVs collected before exercise were as effective at decreasing proliferation as EVs collected after exercise, likely because of the active lifestyles of the participants the EVs came from. All the participants involved in this study were healthy young men, that exercised regularly. Due to this active lifestyle, it is believed that their muscle cells were regularly releasing exerkines through EVs and/or their blood stream already had a high

concentration of EVs containing exerkines. This may account for why pre-exercise EVs caused a decrease in proliferation of the TNBC cell line.

Future research should examine the effects of proliferation on a TNBC cell line using EVs from sedentary participants as well. It is expected that there would be no change in proliferation with pre-exercise EVs, and a decrease in proliferation immediately (IM) after and 24 hr post-exercise time points (Holick *et al.* 2008). This would be due to exerkines only being released post-exercise, unlike what was observed in the active, male participants. Additionally, women should be included in the study to ensure that their exercise-induced EVs contain the same exerkines and have the same effect on proliferation as men's EVs. Different age groups should also be examined to determine if age influences EV contents and its effect on proliferation.

When treating SUM149-PT cells with EVs from before and after exercise, there were minimal changes in proliferation compared to untreated cells. This cell line having minimal changes in cell growth when treated may be due to the different characteristics and molecular mechanisms causing its proliferation compared to BT549 cells. Each cell line is derived from a different person, meaning that the cells have different mutations that drive tumor progression. Additionally, breast cancer cell lines are known to be highly heterogeneous, causing them to have different responses to therapeutic drug treatments. A recent study noted that MDA-MB-231 and MDA-MB-436, two triple negative breast cancer cell lines, are more mechanically different from one another compared MCF10a's, a non-tumorigenic cell line (Shen *et al.* 2020). Although the reason for different cell lines being affected differently by EVs is unknown, further experimentation can be done to help better understand this.

Expression of Epithelial and Mesenchymal Markers

The epithelial to mesenchymal transition (EMT) is a hallmark of cancer progression. Characteristics of mesenchymal cells include increased motility and decreased cell division, which coincides with the decrease in proliferation shown in EV treated cells seen earlier in this study. Cells that are undergoing an EMT are often in cell cycle arrest, which slows proliferation (Akhmetkaliyev *et al.* 2023). E-cadherin, an epithelial marker, plays roles in cell adhesion through the formation of adherens junctions that allow cells to be tightly bound together. N-cadherin, a mesenchymal marker, can induce pathways that lead to increased cell motility (Loh *et al.* 2019).

TNBC cells are known to have increased expression of N-cadherin and decreased expression of E-cadherin, causing a mesenchymal and motile phenotype (Nelson *et al.* 2016). The low levels of E-cadherin expression are likely why there were minimal changes in the amount of E-cadherin expressed in EV treated cells. Treatment of BT549 cells with 24 hr post-exercise EVs caused a significant increase in gene expression of N-cadherin, which is present in mesenchymal cells. Elevated levels of N-cadherin are associated with immunosuppression, allowing cancer cells to escape surveillance of the immune system (Sun *et al.* 2021). The ability to decrease expression of N-cadherin could allow for better efficacy of immunotherapies in cancer patients.

Further research should be done to look at expression of other mesenchymal markers including *Twist1* and Vimentin (*VIM*). A recent study showed that mRNA levels of N-cadherin are increased in cells with vimentin knocked down (Messica et al. 2017). Examining the expression of vimentin in EV treated cells could help determine the cause of increased N-cadherin expression. Additionally, IM post-exercise EVs should be used

to treat TNBC cells to see if there is a difference in gene expression of epithelial and mesenchymal markers compared to 24 hr post-exercise EVs.

Migratory Ability of TNBC Cells When Treated with EVs

TNBC cells are highly migratory, partially due to increased expression of N-cadherin, which allows them to metastasize at higher rates than other types of breast cancer (Lin *et al.* 2008). To examine another biological component and assess the motility of EV treated TNBC cells, migration assays were completed. The assays showed that there was no significant change in migration of post-exercise EV treated cells compared to untreated cells. This may be because BT549 cells are already highly metastatic, allowing high levels of motility (Conner *et al.* 2024). Although N-cadherin is associated with increased motility, it is challenging to examine the difference in migration when cells are already highly migratory.

Another reason there was not a difference in migration may be that changes in migratory abilities require a complete restructuring of the actin cytoskeleton. This involves an alteration of gene expression that lead to different cadherins being expressed and changes in intracellular pathways (Nelson 2008). Observing the effects of one EV treatment after 24 hours may not be enough to cause changes in cadherin expression and function. Future experimentation should examine migration in TNBC cells that are treated with exercise-induced EVs repeatedly, for several days. This is more representative of what would occur in the body of a breast cancer patient that exercises regularly, as their tumor would be repeatedly exposed to exerkines.

Changes in Activation Status of Signaling Proteins

Many signaling pathways affecting cellular activities, such as proliferation, differentiation, and migration are mediated by phosphorylation of proteins. Changes in phosphorylation of these proteins can be observed to determine what pathways are being affected and what downstream affects may be occurring. Phospho-arrays were used to examine changes in phosphorylation of kinases in TNBC cells treated with exercise-induced EVs and better understand what signaling pathways the EVs affected. The phospho-arrays examined two types of kinases, receptor tyrosine kinases (RTKs) as well as intracellular kinases. Several of the kinases showed a significant decrease in phosphorylation when treated with EVs, including GSK-3a/B and ERK1/2. We were interested in confirming these results because these kinases are involved in proliferative pathways. More specifically, phosphorylation of GSK-3a/B can activate pathways that lead to cell division and protein synthesis and ERK is known to have roles in cell migration and proliferation (Figure 12) (Hermida *et al.* 2017). Additionally, AXL had a slight decrease and EGFR had a slight increase in phosphorylation. These RTKs are both known to play significant roles in the progression of TNBC, therefore we wanted to confirm these results as well (Wang *et al.* 2016).

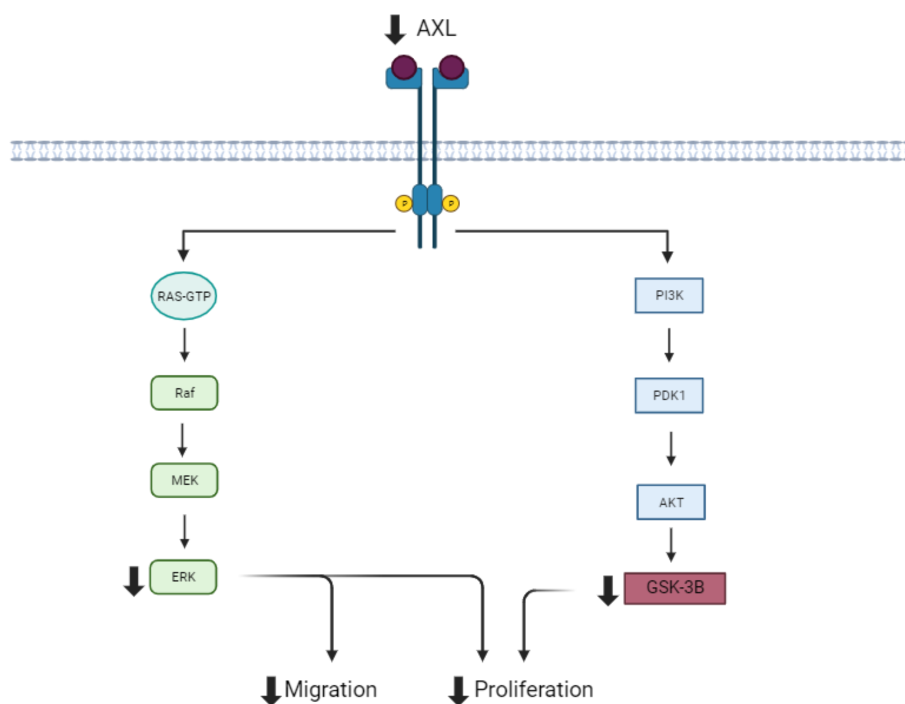

Figure 12. Cell signaling model showing intracellular and downstream effects of decreased phosphorylation of AXL, ERK, and GSK-3B.

Western blots were used to examine pERK, pAXL, pGSK-3a/B, and pEGFR in EV treated TNBC cells compared to untreated cells. Surprisingly, we were unable to confirm the changes in phosphorylation seen in the phospho-array for any of these kinases. All antibodies used have been established in literature in their ability to identify the kinase. Differing results from the phospho-arrays and Western blots may be due to the use of different participants EVs for each experiment. Moving forward, pooling patient samples would be ideal for providing more accurate results.

Further research, utilizing immunoprecipitation pull-downs could be done to more accurately confirm the results seen in the phospho-arrays. This assay has a more similar procedure to a phospho-array and would allow for enrichment of the protein signal. Additionally, inhibitors of these pathways could be used to further confirm and specify

the effects of these kinases on EV treated TNBC cells. Overall, this will allow for a better understanding of the roles signaling proteins play in the decrease in proliferation of EV treated TNBC cells seen in this study.

Apoptotic Markers in EV Treated Cells

The presence of cleaved caspase-3 and PARP were examined through a Western blot to determine if EV treatment was inducing apoptosis in BT549 cells. If apoptosis was occurring, we expected the presence of cleaved caspase-3 and cleaved PARP. Bands for cleaved caspase-3 were unable to be visualized, possibly due to minimal amounts of the protein within cells. PARP was present but not in the cleaved form, signifying that cells may not be undergoing apoptosis.

Flow cytometry could be completed to confirm these results, looking for the presence of apoptotic cells. Forward (cells size) and side scatter (granularity, internal complexity of a cell/cellular components) are collected to determine cells size and shape. Additionally, fluorescent labels can be added to the cells to detect apoptosis by determining whether the cell membrane is intact or not. Annexin V binds to phosphatidylserine, which is a marker of apoptosis when it is located on the outer leaflet of the plasma membrane. Propidium iodide (PI) is an impermeable nuclear dye that fluoresces when it interacts with DNA or RNA. The presence of Annexin V without PI indicates that the cells are in early apoptosis and the presence of both Annexin V and PI indicates that the cells are undergoing necrosis/late apoptosis (Novus Biologicals, 2023).

Further Research

The duration of EV treatment on TNBC cells was considered while planning this study and is a factor that we intend to examine further. Based on previous research, EVs are taken up by cancer cells in the first 24 hours of treatment, indicating that a shorter incubation time may be necessary to examine the effects of these EVs on TNBC cells (Franzen *et al.* 2014). Changing the incubation period from three days to one day may cause different results to be produced from each of these experiments.

An alternative mechanism that may be causing the decrease in proliferation of BT549 cells treated with exercise-induced EVs is muscle derived cytokines. EVs are known to carry these proteins that can stimulate the immune system and aid in the prevention and treatment of muscular diseases, infections, and cancer (Vitucci *et al.* 2023). Several studies have determined what cytokines are present in exercise-induced EVs as well as their effects on cancer cells. Cytokines such as IGF-1, IL-6, IL-10 and OSM are capable of decreasing proliferation of cancer cells with IL-10 and OSM specifically decreasing proliferation in a breast cancer cell line (Hojman *et al.* 2011; Hekmatikar *et al.* 2023).

To better understand the mechanism allowing EVs to change proliferation of TNBC cells, EVs should be sequenced to determine their contents (nucleic acids and proteins). Many miRNAs have been previously identified in exercise-induced EVs that play roles in cancer progression and patient prognosis. Identification of miRNAs present in exercise-induced EVs can be used to better understand the mechanisms allowing EVs to decrease phosphorylation of a TNBC cell line. Additionally, inhibitors of miRNAs can

be used with Western blots to determine if specific miRNAs are causing the decrease of phosphorylation in signaling proteins observed in the phospho-arrays.

Cell lines are widely used in basic cancer research due to their cost-effectiveness and ability to grow indefinitely. Although tissue culture is a known starting point for many research projects, it is not the most accurate representation of how a tumor in a patient would respond to exercise-induced EVs. Animal models using xenografts, patient derived tumors, would likely give the best representation of the effects of EVs on TNBC cells. Unfortunately, due to time and monetary constraints, this method was not utilized for this study, but should be examined in the future.

Conclusions

Data from this study has provided insight into the effects of exercise-induced extracellular vesicles on a TNBC cell line, including changes in proliferation, activation status of signaling proteins, and gene expression of differentiation markers. Based on our results, it is likely that many pathways are being affected by the EVs to cause a decrease in proliferation. Additional research could provide an understanding of the mechanisms that allow exercise to slow tumor growth and progression in breast cancer patients. The hope is that this leads to a more effective and minimally invasive treatment method for TNBC.

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APPENDIX A

PROLIFERATION ASSAY DATA

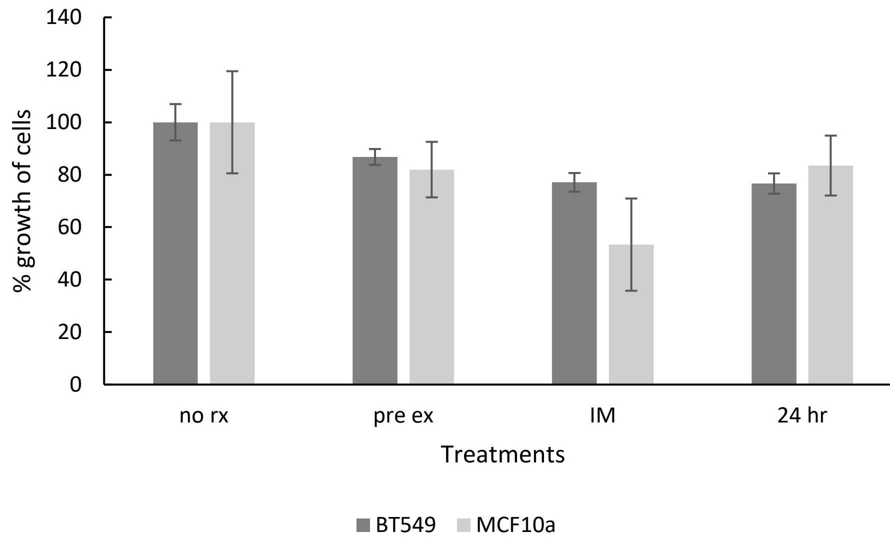

Figure 13. Percent cell growth of BT549 and MCF10a cell lines when treated with 100 uL of EVs from three time points (pre-exercise, IM post, and 24 hr post exercise), in comparison to untreated cells (no rx). Based on averages from proliferation assays using one participants EVs (n=3), including standard deviation.

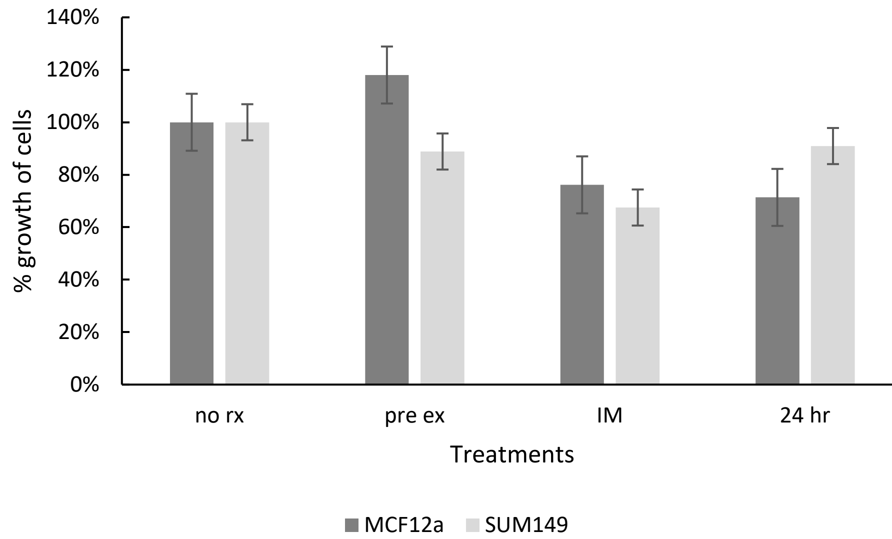

Figure 14. Percent cell growth of SUM149-PT and MCF12a cell lines when treated with 100 uL of EVs from three time points (pre-exercise, IM post, and 24 hr post exercise), in comparison to untreated cells (no rx). Based on averages from proliferation assays (n=2), including standard deviation.

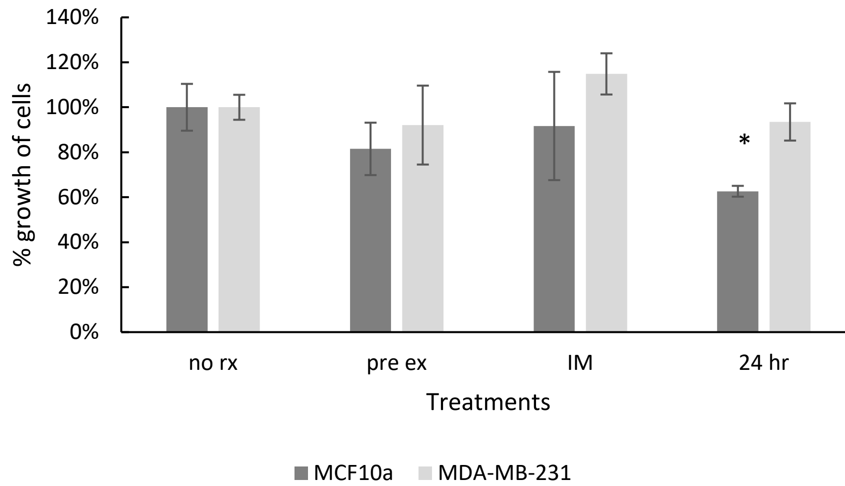

Figure 15. Percent cell growth of MDA-MB-231 and MCF10a cell lines when treated with 100 uL of EVs from three time points (pre-exercise, IM post, and 24 hours post exercise), in comparison to untreated cells (no rx). Based on averages from proliferation assays (n=2), including standard deviation. *=$p \leq 0.05$, t test.

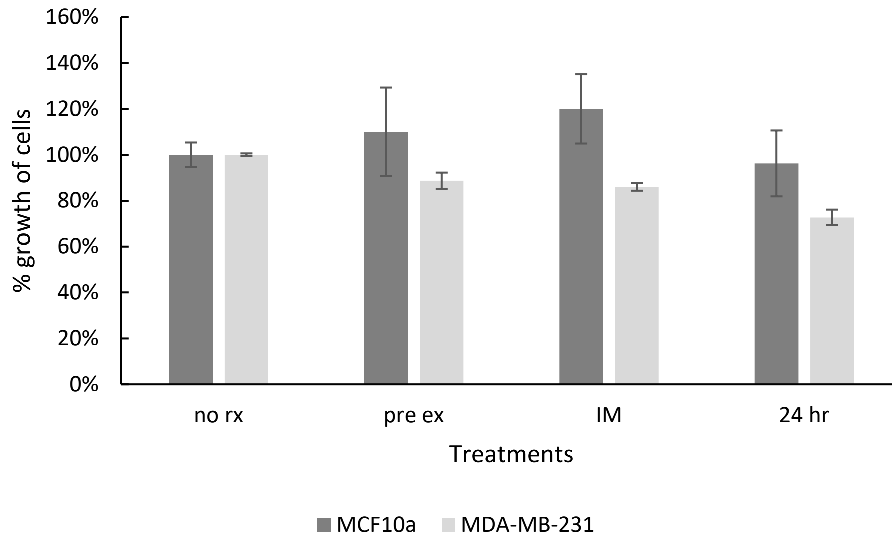

Figure 16. Percent cell growth of MDA-MB-231 and MCF10a cell lines when treated with 50 uL of EVs from three time points (pre-exercise, IM post, and 24 hours post exercise), in comparison to untreated cells (no rx). Based on averages from proliferation assays (n=2), including standard deviation.

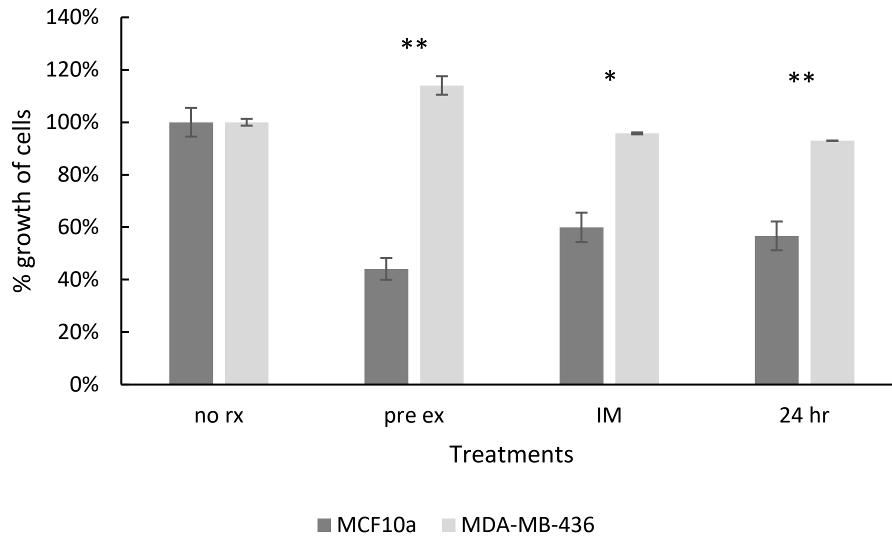

Figure 17. Percent cell growth of MDA-MB-436 and MCF10a cell lines when treated with 100 uL of EVs from three time points (pre-exercise, IM post, and 24 hours post exercise), in comparison to untreated cells. Based on averages from proliferation assays (n=2), including standard deviation. *=p≤0.05, t test. **=p≤0.005, t test.

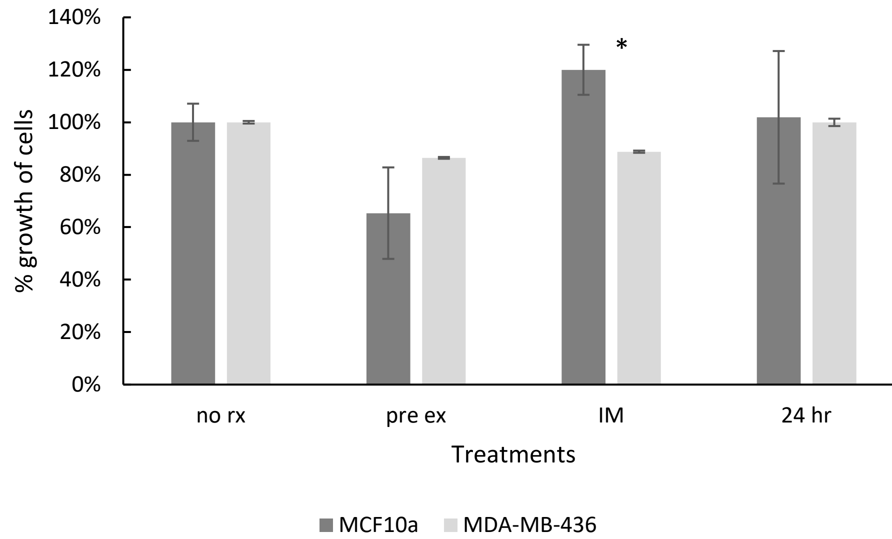

Figure 18. Percent cell growth of MDA-MB-436 and MCF10a cell lines when treated with 50 uL of EVs from three time points (pre-exercise, IM post, and 24 hours post exercise), in comparison to untreated cells. Based on averages from proliferation assays (n=2), including standard deviation. *=p≤0.05, t test.

APPENDIX B

PHOSPHO-ARRAY RAW DATA

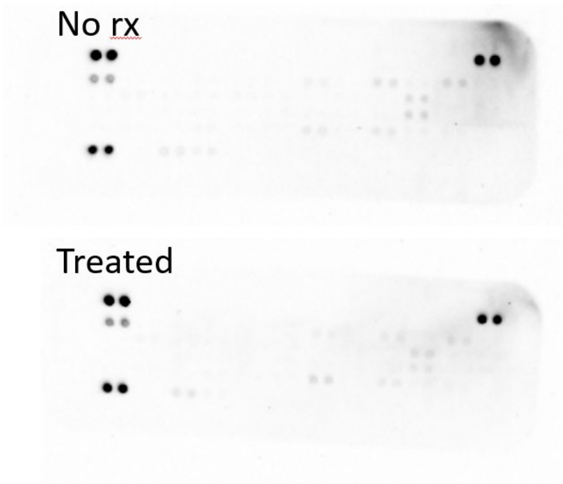

Figure 19. Phospho-RTK array comparing IM post exercise EV treated and untreated BT549 cells.

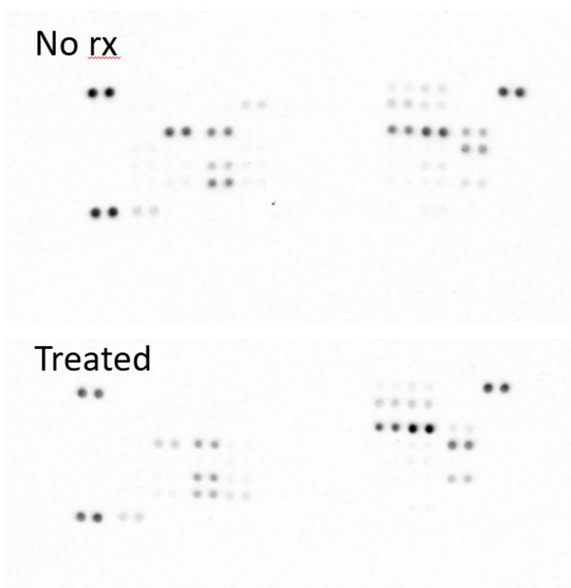

Figure 20. Phospho-kinase array comparing IM post exercise EV treated and untreated BT549 cells.

APPENDIX C

PHOSPHO-ARRAY QUANTIFICATION

Table 1. Average percent change in phosphorylation of RTKs, comparing IM post-exercise EV treated and untreated BT549 cells (Rauch *et al.* 2011).

RTK	Function	Average percent change
EGFR	Cell survival and growth	26.21
AXL	Proliferation, migration, differentiation	-21.19
Eph R (EphA2)	Cell motility	-23.8
ROR (ROR1)	Cell migration	27.97
Insulin R	Cell death	-31.93
DDR2	Tissue remodeling	28
Insulin R (ALK)	Cell death	39.57
Eph R (EphB3)	Tumor metastasis	16.72

Table 2. Average percent change of phosphorylation of intracellular kinases, comparing IM post-exercise EV treated and untreated BT549 cells (Rauch *et al.* 2011; Hermida *et al.* 2017; Fenton and Gout 2011; Lv *et al.* 2017).

Kinase	Function	Average percent change
B-catenin	EMT, proliferation	-7.69
GSK-3a/B	Cell cycle, development, proliferation	-361.95
WNK-1	Ion transport	-81.11
PLC-y1	Increases intracellular Ca2+	43.44
GSK-3B	Inflammation, glucose metabolism	-42.36
ERK1/2	DNA repair, cell cycle, proliferation	-186.96
P70	Cell cycle regulation	17.28
P70	Cell cycle regulation	31.23
RSK1/2/3	Proliferation and motility	31.85
PRAS40	Proliferation	-203.7
Chk-2	Cell cycle arrest and apoptosis	11.72
C-Jun	Proliferation, apoptosis, development	60.63
Akt1/2/3	Cell survival, proliferation, apoptosis, migration	-33.33
STAT3	Cell growth and survival	47.76

APPENDIX D

WESTERN BLOTS CONFIRMING PHOSPHO-ARRAYS

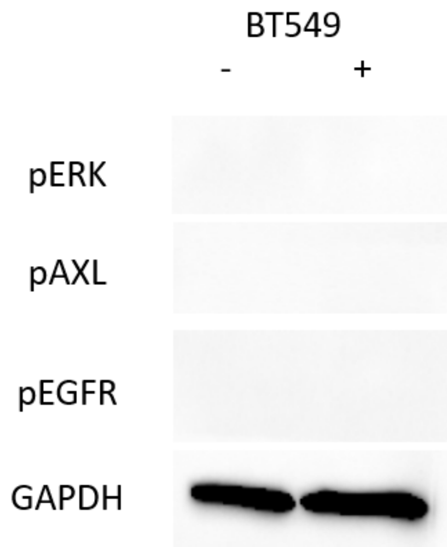

Figure 21. Western blot analysis examining the presence of pERK, pAXL, and pEGFR in BT549 cells treated with exercise induced EVs (IM post exercise) or untreated (no rx). GAPDH served as the loading control.

APPENDIX E

WESTERN BLOT OF APOPTOTIC INDICATORS RAW DATA

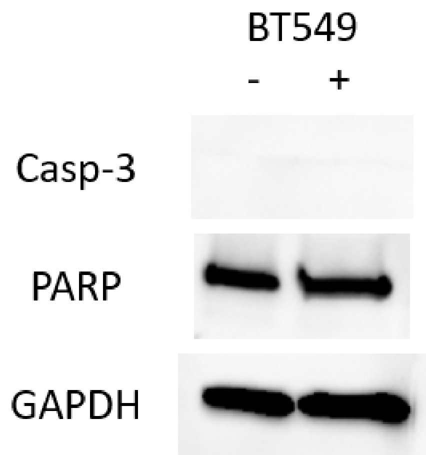

Figure 22. Western blot analysis examining the presence of caspase-3 and PARP in BT549 cells treated with EVs (IM post exercise) or untreated (no rx). GAPDH served as the loading control.