Embryonic Stem Cell-Derived Exosomes Increase the Antiproliferative Activity of Doxorubicin in Breast Cancer

Alexander M. Hirsch
Virginia Commonwealth University

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Embryonic Stem Cell-Derived Exosomes Increase the Antiproliferative Activity of Doxorubicin in Breast Cancer

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By
Alexander M. Hirsch
M.A., Yale University, 2014

Advisor: Dr. Rakesh C. Kukreja
Department of Internal Medicine

Virginia Commonwealth University
Richmond, Virginia
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Abstract

The field of cancer research has grown immensely in recent decades and has led to a better understanding of the causes of the disease, as well as greatly improved treatment for various types of cancers, especially breast cancer. One of the most effective treatments involves the chemotherapeutic drug doxorubicin (DOX). DOX is an effective tool against all types of breast cancer, especially against triple negative breast cancer. However, DOX causes adverse side effects that include damage to the heart and skeletal muscle, particularly above specific cumulative doses. Recent evidence suggests that embryonic stem cell-derived (ES) exosomes, nanoscale extracellular vesicles that carry proteins, messenger RNA, and microRNAs, may be able to mitigate some of the cardio- and cytotoxic effects of DOX without reducing its efficacy.

The present study examined the effects of combined treatment with DOX (1 μM) and ES exosomes (10 μg/mL) on three cancer cell lines, MCF7, MDA-MB-231, and MDA-MB-468. The DOX/ES exosomes treatment increased cell death and increased apoptosis specifically compared to control, as measured via dye exclusion assay and flow cytometry. The treatment also decreased cell growth compared to control, as measured via MTS cell proliferation assay. In addition, DOX/ES exosomes treatment also increased expression of pro-apoptotic Bax while decreasing the expression of anti-apoptotic Bcl-2, as measured via Western blot. Finally, the DOX/ES exosomes treatment decreased expression of miR-200c, a microRNA associated with preventing epithelial-mesenchymal transition, a process that is integral to metastasis.

Although increased cell death and apoptosis and decreased cell proliferation implies that the DOX/exosomes treatment is effective against cancer, the decrease in miR-200c expression may suggest the opposite and will be investigated further in future studies. Even so, the results of
this study suggest that exosomes may be an important component to reduce the harmful effects of cancer treatment in the future.
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1. Introduction

All living things exist as a constant balance of growth and death. During adulthood, healthy humans enjoy an even balance between cell proliferation and cell death, but during certain stages of life, and during certain disease states, proliferation can greatly outpace death. In the developing embryo, cell division occurs at a rapid pace to meet the needs of the growing organism. At the same time, certain highly specific populations of cells die off to allow organs to take certain shapes or to promote the formation of channels and orifices. For example, the fetal heart develops as two separate endocardial tubes. As the surrounding cells grow or die off, these tubes are pushed closer and closer together until they fuse, a feat accomplished by the death of cells in the tubes’ walls (van den Hoff et al., 2000). Cardiac development continues to rely on strategic growth and death as the now-fused vessel twists and segments, eventually forming the mature four-chambered heart.

The same processes that allow for rapid fetal growth, however, can also lead to harmful or even fatal consequences. Cancer is one such outcome. Essentially, cancerous growth occurs when a population of cells loses its growth inhibitions and multiplies out of control. Cells that would normally divide slowly now replicate at a rapid clip, and the processes that would normally cause cells in such a frenzy to commit suicide rather than endanger the larger organism fail to activate. As they divide, these aberrant cells continue to accumulate genetic damage, allowing them to change shape, leave their anchor points, and establish new colonies elsewhere in the body, a process called metastasis.

This paper will begin with background information on cancer, including the process of cancer transformation and characteristics important to all cancers. I will then discuss breast cancer specifically, as well as strategies to combat its growth and spread. Next, I will elaborate
on the process of cell death, the intended consequence of cancer treatment, and its various forms and I will consider the risks of administering treatment that causes it. I will review the properties of exosomes, acellular vesicles released by many cell types, and their implications on cancer treatment. Finally, I will discuss the current study, its results, and the conclusions drawn from those results.

1.1. Challenges of Cancer Research

Studying cancer cells is challenging for a variety of reasons, not least because they defy easy classification. First, it was assumed that primary tumors—that is, tumors present within an organism from which a cancer cell line may be isolated—begin from a singular cell or group of clonal cells and then grow to form a larger mass. Although some tumors likely are the result of a single (or group of clonal) mutated cells, evidence suggests that many are not (Parsons, 2008). Instead, tumors can form as a result of disrupted cell signaling processes caused by, for example, inflammation and its resulting destruction to the extracellular matrix (ECM) and invasion of immune cells. Epithelial cells attached to the ECM may then sustain genomic damage that then spirals into tumor formation. Alternatively, inflammatory processes may provoke cells with existing precancerous abnormalities, either genetic or epigenetic, into cancerous proliferation (Bissell & Radisky, 2001). Such circumstances suggest the possibility that distinct, neighboring cells may be affected en masse by a single precipitating event, which would cause them to incorporate into a single, polyclonal tumor.

By itself, many tumors’ polyclonal origins make propagating a cell line from individual, “representative” cells quite difficult. However, even monoclonal tumors are quite heterogenous (Loeb, Loeb, & Anderson, 2003). A tumor can have similar or even greater complexity to a normal organ: many different cell types comprise a single tumor, including cancer cells, cancer-
associated fibroblasts (CAFs), cancer stem cells (CSCs), immune inflammatory cells, endothelial cells (that form the tumor vasculature), and associated pericytes (Hanahan & Weinberg, 2011). All six of these cell types work together to sustain the tumor as a whole and thus none are totally representative of the tumor in isolation. What is more, many cancer cell lines used in research were not originally derived from a solid tumor but were isolated from metastases present in ascites fluid or pleural effusion (in the case of the MCF7 line, for example) (Lacroix & Leclercq, 2004). These cells have already undergone various changes to facilitate metastasis, including the process of epithelial-mesenchymal transition (EMT), and therefore may be yet another step removed from the tumor in which they originated (Birchmeier & Behrens, 1994).

1.2. Genetic Foundations of Cancer

Despite the difficulties inherent in studying cancer, many of its properties have been elucidated over the past few decades. Before a deeper discussion of the characteristics that underly the formation and growth of tumors, it is important to discuss the concept of oncogenes and tumor suppressor genes and their involvement in tumor formation.

Certain genes—proto-oncogenes and tumor suppressor genes—have a special relationship with cancer growth and development. Mutations in both types of genes can lead to the induction of cancer, though in opposite ways. First, genes that can be classified as “proto-oncogenes” are involved in pathways that control cell growth. In their normal state, they ensure that cells that divide to replenish cells lost through normal means—especially epithelial cells like those in the skin or gut lining—will continue to do so. However, if a cell develops a mutation to a proto-oncogene that circumvents the normal safeguards that keep cell growth in check, the proto-oncogene becomes an oncogene and the cell may begin to divide too rapidly. As the growing population of oncogene-containing cells accumulates further mutations, the cells can
form a tumor. Because the formation of an oncogene represents a gain-of-function mutation, only one copy need be damaged to promote cell proliferation.

On the opposite side of the coin, tumor suppressor genes code for proteins that limit cell growth. These genes keep actively dividing cells from dividing too fast by acting as checks on the cell cycle, growth signaling, or genetic integrity. They also function in mature cells to maintain their differentiated state. If a tumor suppressor gene is damaged, the cell may begin to grow out of control or rapidly accumulate genetic damage. Both circumstances promote the formation of a tumor. However, because a mutated tumor suppressor gene represents a loss-of-function mutation, both copies of a given gene must be mutated to achieve the maximum carcinogenic power. Of course, because tumor suppressor genes and proto-oncogenes both help to prevent cancer when functioning normally, damage to both types of genes greatly increases the likelihood of a malignancy (Lodish et al., 2000).

1.3. Cancer’s Defining Characteristics

The genetic changes that give rise to cancer describe discrete events, but cancer itself is more than the sum of its genetic parts. Luckily, the past decades have given us a solid framework upon which to build an understanding of the myriad mechanisms underlying cancer genetics and signaling. Hanahan and Weinberg (2000) described seven hallmarks of cancer that are necessary for its formation and proliferation. Later, the researchers added two more hallmarks based on research accomplished in the intervening years (Hanahan & Weinberg, 2011). I will describe each hallmark briefly.

1.3.1. Growth signal self-sufficiency. First, cancer must be self-sufficient in growth signals. Normal human cell growth is regulated by many different growth signals that govern whether and how quickly cells will divide and how less-differentiated cells will change to
become mature, differentiated cells. Cancer cells have broken out of this paradigm. Some are able to manufacture signals that stimulate their own growth (Sporn & Todaro, 1980). In other cases, the receptors that would bind growth factors are overexpressed, allowing more receptors to be activated by the same signal. In addition, increased interaction between receptors ultimately results in increased growth factor sensitivity (Slamon et al., 1987; Yarden & Ullrich, 1988). Finally, a genetic mutation can alter the structure of a receptor in a way that allows it to fire constitutively, even in the absence of the growth factor stimulus (Bishayee, 2000).

1.3.2. **Resistance to anti-growth signals.** To succeed, cancer cells must not only establish their independence from growth signals but also develop resistance to anti-growth signals. Often, this means developing a means to bypass cell cycle checks that would normally cause maturing cells to arrest in the G0 or G1 phases. The main regulators of progression from G1 into S phase are the retinoblastoma protein (pRb) and the related proteins p107 and p130. The transcription factor E2F is responsible for controlling expression of many genes necessary to advance through the cell cycle; pRb sequesters E2F, halting the cell cycle, but phosphorylation of pRb releases E2F, allowing the cell to proceed through the cell cycle. Transforming growth factor β (TGFβ), a cytoplasmic signaling molecule, blocks pRb phosphorylation, preventing the cell from leaving G1 and therefore halting proliferation (Weinberg, 1995). TGFβ is therefore an anti-growth signal. If TGFβ activity is disrupted—for example through mutations to TGFβ receptors (Markowitz et al., 1995), mutation (Schutte et al., 1996) or deletion (Chin, Pomerantz, & DePinho, 1998) of its downstream targets, or deletion of pRb itself or sequestration by viral oncoproteins (Dyson, Howley, Münger, & Harlow, 1989)—the cell can bypass the G1 checkpoint and continue to proliferate.
1.3.3. Resistance to death signals. Even if nascent cancer cells can grow without growth signals and ignore anti-growth signals, they must also be able to avoid death signals. In normal cells, removal of an important growth factor—e.g. testosterone for prostate gland cells—or addition of certain stimuli—e.g. glucocorticoid hormones for immature thymocytes—can cause apoptosis, a form of programmed cell death (Alison & Sarraf, 1992), as can a variety of other more specific stimuli. The process of apoptosis itself is discussed in greater detail below, but to give a brief description, a cell that has received an apoptotic signal begins to shrink as the cell’s components are degraded into fragments. The fragments are packaged into membrane-bound compartments that break apart from one another in a process called blebbing. The vesicle-like apoptotic bodies are then subsumed by phagocytic cells and degraded (Kerr, Wyllie, & Currie, 1972).

Cell death in tumors is a major determinant of the tumor’s growth rate (Alison & Sarraf, 1992). As such, the capacity to avoid apoptosis is essential for a tumor to continue to grow. Cancer cells can avoid apoptosis (and other forms of cell death) by several means. A tumor suppressor gene, TP53, the gene that encodes the protein p53, is mutated across various forms of cancer. In fact, roughly half of all tumors diagnosed each year contain TP53 mutations (Harris, 1996; Levine, 1997). The protein p53 is an important signaling intermediary to several pathways, most notably apoptosis. As mentioned above, apoptosis can be caused by various stimuli. However, many of the signaling pathways involved funnel through p53, meaning that the loss of p53 or expression of an aberrant protein abrogates the apoptotic effect of several different signals, including hypoxia, oncogene overexpression, and DNA double-strand breaks (Levine, 1997). As a result, cells with defective or missing p53 are susceptible to further perturbation of signaling pathways and are less likely to undergo “altruistic suicide” via apoptosis.
1.3.4. Immortalization. Although the above hallmarks of cancer—growth signal independence, resistance to anti-growth signals, and resistance to apoptosis—lay the groundwork for unrestrained proliferation, there are still more safeguards that prevent unlimited growth. Normal cells in culture—and likely in vivo as well—will only go through a finite number of replications before they lose their replicative ability, a state termed “senescence” (Hayflick, 1997). This phenomenon is attributed to the progressive shortening of telomeres, repeating regions at the end of the chromosome that seem to protect from excessive recombination or even end-to-end fusion of one chromosome to the next. During normal DNA synthesis, telomeres lose approximately 65 bp per replication (Counter et al., 1992). However, disabling certain tumor suppressor genes—for example, RB (the gene encoding the pRb protein) and TP53 in cultured human fibroblasts—allows cultured cells to continue replicating past the point at which they would ordinarily enter senescence. If allowed to continue replicating, the altered cells will eventually reach a crisis point at which a massive number of cells will die off, leaving behind only a small number of survivors (1 in \(10^7\)). The cells that remain are immortal, able to replicate indefinitely (Wright, Pereira-Smith, & Shay, 1989).

On a molecular level, cancer cells employ one of two strategies to continue replicating past the normal senescence cutoff. The vast majority upregulate telomerase, a reverse transcriptase that adds hexameric repeats to the ends of the telomeres, which allows cells to replicate with no net loss of telomeric DNA (Bryan & Cech, 1999; Counter et al., 1992). Other surviving cells do not express telomerase but instead maintain their telomeres through homologous recombination (Bryan, Englezou, Gupta, Bacchetti, & Reddel, 1995; Cesare & Reddel, 2010; De Vitis, Berardinelli, & Sgura, 2018). Regardless of the method, maintaining telomere length seems to be required for cellular immortalization.
1.3.5. **Angiogenesis.** Like normal tissues, cancer cells must be supplied with oxygen and nutrients and must be able to dispose of waste to survive (albeit at different rates than normal cells). Therefore, tumors must be able to recruit blood vessels to grow past a microscopic size. In normal adult tissue, vascular growth—angiogenesis—is only active during processes like wound healing or menstrual cycling (Bouck, Stellmach, & Hsu, 1996). Pre-malignant cells initially lack the ability to attract new blood vessels. As they transform into malignant tumors, they quickly develop the capacity to induce angiogenesis (D. Hanahan & Folkman, 1996).

Angiogenesis is governed by multiple factors, both pro- and anti-angiogenic. Thrombospondin-1 (TSP-1) was the first discovered and is perhaps the best known anti-angiogenic factor (Good et al., 1990). Many other anti-angiogenic factors are derived from larger proteins with functions unrelated to angiogenesis and include angiostatin and other “statins” derived from collagens. On the other hand, pro-angiogenesis factors tend to be receptor tyrosine kinase ligands and include vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) (Bergers & Benjamin, 2003). In normal cells, stimuli such as the hypoxic environment that develops around a wound can induce angiogenesis to promote wound healing, but once the wound has closed and the oxygen supply has returned to the healing tissues, angiogenesis ceases (Knighton, Silver, & Hunt, 1981). As a cell transforms, mutation or loss of certain tumor suppressor genes or induction of oncogenes can begin to tilt the balance of signals toward pro-angiogenesis. Also, as a tumor grows larger, the physical mass and size begin to restrict oxygen delivery and waste removal by native blood vessels, stimulating pro-angiogenic processes. This tilt towards pro-angiogenic signaling is often referred to as the “angiogenic switch” that allows cancer cells to continue to proliferate (Dor, Porat, & Keshet, 2001). In some cases, the angiogenic switch can be
flipped indirectly when a protein regulating an angiogenesis regulator is overexpressed or
downregulated itself. For example, p53, a master tumor suppressor gene discussed above with
respect to apoptosis, also acts as a transcription factor to positively regulate TSP-1 synthesis. If
p53 is lost due to mutation or another means, expression of TSP-1 will decrease. Because TSP-1
inhibits angiogenesis, the loss of this protein allows the balance to tip toward pro-angiogenesis
and furthers tumor growth (Dameron, Volpert, Tainsky, & Bouck, 1994).

1.3.6. Metastasis. Tumor growth by itself presents a challenge to the system that the
tumor has colonized. Any tumor will use up extra energy and encroach on nearby tissues, and
many tumors produce the same signaling proteins as their tissues of origin, potentially interfering
with hormonal balances or other signaling systems. However, tumors that remain encapsulated
and are not near a major vessel or structure that might be compressed and damaged are generally
benign and can be treated and cured (Talmadge & Fidler, 2010). To become malignant, tumors
must be able to metastasize. That is, cells must leave their site of origin, travel through the body
(usually through the vasculature or lymphatic system), and establish a new colony elsewhere.
Unlike many of the hallmarks discussed above, metastasis most often occurs later in tumor
development: the likelihood of metastasis tends to corelate with tumor size, meaning that smaller
tumors generally (but not always) have not metastasized by the time they are detected, while
larger tumors are often already metastatic (Talmadge & Fidler, 2010).

Metastasis tends to proceed through a series of discrete steps. Once a tumor has
established itself and undergone vascularization, cells near blood or lymphatic vessels must
penetrate the basement membrane surrounding the tumor and then that surrounding the vessel in
question. The migrating cells then pass through the vessel wall in a process known as
transendothelial migration or intravasation and travel through the bloodstream or lymphatics,
where they are known as circulating tumor cells (CTCs) (Eddy, Weidmann, Sharma, & Condeelis, 2017; Nieto, Huang, Jackson, & Thiery, 2016). Metastatic cells of a given origin will tend to colonize specific tissues, according to the “seed and soil” hypothesis first proposed in 1889 (Paget) and born out in more recent years (Fidler, 2003). In fact, it has become increasingly clear that metastatic tumors induce a “pre-metastatic niche” through system-wide release of tumor-secreted factors and extracellular vesicles. The pre-metastatic niche exists as a preset environment awaiting the arrival of a CTC (Peinado et al., 2017). Therefore, once a CTC reaches a tissue containing such a niche, it can leave the vessel within which it traveled through a process called extravasation and travel to the site of colonization. To reach what will become the metastatic niche, the CTC must break through the extracellular matrix (ECM) surrounding the organ to be colonized. To do this, cancer cells employ protrusions called invadopodia that attach to and degrade the ECM (Liotta, 1986; Murphy & Courtneidge, 2011). At this point, the cell has bypassed the body’s safeguards against metastasis and can begin to establish a new tumor distant from its site of origin.

1.3.6.1. Epithelial-mesenchymal transition. Aside from the various adaptations cancer cells need to develop to be able to successfully travel from the tumor site to the metastatic niche, metastatic cells also need to undergo a transformation from attached, cuboidal or squamous epithelial-like cells to dedifferentiated, front-back polar mesenchymal-like cells (Nieto et al., 2016). The process governing this transformation is referred to as epithelial-mesenchymal transition (EMT). At the other end of the journey, the reverse process, mesenchymal-epithelial transition (MET) primes the metastatic cell for cell-cell attachment and other necessities for tumor formation. EMT relies on a variety of stimuli, including protein transcription factors (referred to as EMT-TFs), microRNAs, and epigenetic and post-transcriptional regulators.
Though a multitude of factors control the EMT process, the most commonly recognized markers of epithelial-like cells are E-cadherin, occludins, and cytokeratins, while the canonical markers of mesenchymal-like cells are N-cadherin and vimentin (Thiery, Acloque, Huang, & Nieto, 2009).

1.3.7. **Metabolic modifications.** In addition to the longer-established characteristics of cancers described above, more recent research has brought additional characteristics to light. Like many aspects of cancer development, tumor metabolism is quite unlike that of normal cells. Instead of utilizing the citric acid cycle and oxidative phosphorylation in the presence of oxygen to generate ATP from glucose and other glycolytic molecules, many forms of cancer preferentially employ lactic acid fermentation. Although the anomalous nature of cancer cell metabolism was observed as long ago as 1927 (Warburg, Wind, & Negelein), it was not clear for many years why cancer cell metabolism should differ so drastically from that of normal cells. Especially when one considers the rapid rate of growth of malignant tumors, it would seem counter-productive to employ a form of energy conversion that is grossly inefficient at producing ATP compared to the alternative. Recent interest in cancer metabolism from a therapeutic perspective has driven new inquiry into Warburg’s original observation. In particular, there has been renewed attention to the hypothesis that cancer cells employ lactic acid fermentation in order to preserve carbon skeletons for use in macromolecule synthesis (nucleotides, amino acids, lipids, etc.) (Vander Heiden, Cantley, & Thompson, 2009). Stranger still, tumors have been documented that contain two populations of cells that use opposite metabolic strategies. In these tumors, one population of cells, deprived of oxygen by their distance from the blood supply, employs glycolysis as described above and releases built-up lactate. The other, by virtue of its
proximity to a capillary, takes in the released lactate and generates ATP via oxidative phosphorylation (Feron, 2009; Semenza, 2008).

Interestingly, the human placenta utilizes a similar metabolic strategy to cancer’s “aerobic glycolysis.” However, instead of regenerating NAD⁺ by converting glucose to lactic acid, NAD⁺ is regenerated via the polyol pathways (Burton, Jauniaux, & Murray, 2017), bolstering the idea that cancer does not create novel pathways but instead subverts extant strategies for its own purposes.

1.3.8. Immune system evasion. Finally, cancer cells’ ability to evade the immune system has been highlighted recently as a possible line of attack to combat the growth of tumors. Much energy has gone into the idea that the immune system itself can be tuned to recognize cancer cells by the strategies they use to avoid detection (Yousefi, Yuan, Keshavarz-Fathi, Murphy, & Rezaei, 2017). Indeed, the ability to avoid immune detection and response seems to be a critical determinant of which cancer cells survive and proliferate and which are killed off before they can take root.

The concept of immune surveillance, the idea that the immune system monitors neoplastic activity and kills off the vast majority of cancerous cells before they can form tumors, has existed since at least 1909 (Ehrlich). However, evidence for the hypothesis was not available until the 1950s, and the ensuing years saw the growth of research that seemed to discredit immune surveillance entirely (Dunn, Bruce, Ikeda, Old, & Schreiber, 2002; Ribatti, 2017). Nevertheless, in the past two decades new research has started to back up immune surveillance. Experiments on mice lacking critical components of the immune system have shown an increase in cancers not caused by viral infection in these mice, and observational experiments of immunocompromised humans have shown similar results (Dunn et al., 2002). Furthermore,
experiments in which tumors were transplanted between wild-type and immunocompromised mice and vice versa have shown that tumors grown in animals lacking an immune system are more immunogenic than those from immunocompetent individuals. These results suggest that many of the tumors that form in the absence of an immune system would have been weeded out in healthy individuals (Shankaran et al., 2001). Although there are still many questions surrounding the role of the immune system in fighting cancer, researchers have generally accepted the idea that cancer must evade the immune system as a prerequisite for successful growth (D. Hanahan & Weinberg, 2011).

1.4. Breast Cancer: Description and Treatment

Now that I have laid out the defining characteristics of cancer as they are currently understood, I will shift my focus to breast cancer specifically. Breast cancer has been recognized as a distinct disease since antiquity; descriptions date as far back as the ancient Egyptians circa 1600 BCE (Breasted, 1930). Today, breast cancer can be divided into three subtypes based on expression of three cell-surface receptors: the estrogen receptor (ER), the progesterone receptor (PGR),\(^1\) and a third receptor known as HER2 (human epidermal growth factor receptor 2) or erbB2.\(^2\) The first subtype, defined by tumors that express ER and PGR and do not overexpress HER2, is also the most treatable and includes approximately 70% of patients. The second subtype consists of tumors that overexpress HER2 (15-20% of patients), and the third consists of tumors that express none of the three markers (10-15% of patients) and is commonly referred to as “triple negative” breast cancer (TNBC). TNBC is the most dangerous subtype: it is most likely to recur, has the worst 5-year survival rate (85% versus 94% and 99% for ER/PGR positive and

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\(^1\) ER and PGR are collectively referred to as HR, hormone receptors.
\(^2\) HER2/erbB2 has no ligand-binding domain (Zurrida & Veronesi, 2015).
HER2 positive, respectively), and has the worst median overall survival rate (1 year versus 5 years for both other subtypes) (Waks & Winer, 2019).

Tumor subtype is relevant not only to develop an accurate prognosis but also to determine the appropriate systemic therapy for non-metastatic cancers. Patients with HR positive tumors can be treated with hormone therapy along with chemotherapy in some cases. Patients with HER2 positive tumors are treated with HER2-targeted antibodies or small-molecule inhibitors combined with chemotherapy. Finally, TNBC can only be treated with chemotherapy (barring the development of new precision-targeted treatments) (Waks & Winer, 2019).

Unlike HR positive or HER2 positive tumors, no specific therapeutic targets have been identified for TNBC. In addition, TNBC tends to “escape” from conventional treatments and form metastases (Khaled & Bidet, 2019; Perou et al., 2000; Sørlie et al., 2001). As such, there has been recent interest in classifying TNBC more extensively via gene expression patterns and epigenetics. Doing so in a clinical setting is becoming more feasible as costs decrease, but the science is not yet well-developed (Zugazagoitia et al., 2016).

1.4.1. Treatment of breast cancer. Having described the basic characteristics unique to breast cancer, I will now discuss the five forms of treatment currently in use. Surgery is the oldest treatment, but surgical procedures have also changed over the years, gradually shifting from radical excision to more conservative practices. Evidence has emerged in favor of breast-conserving surgeries, especially for smaller tumors. Larger or more numerous tumors still require mastectomy, though often without the removal of the underlying muscle. In the past, complete axillary dissections were performed to remove possible metastases to the lymph nodes. Current techniques allow for biopsy of only the lymph nodes with the shortest connection to the tumor
Radiation therapy has long been used in conjunction with surgery, especially breast-conserving surgery, and nowadays is often performed intraoperatively. Unfortunately, radiation therapy can adversely affect the heart (for left-sided tumors) and the lungs (Zurrida & Veronesi, 2015).

While tumor subtype is less important for the efficacy of surgery or radiation therapy, hormone therapy is quite effective against HR positive tumors but is ineffective against tumors that do not express ER or PGR. Predictably, hormone therapy interacts with a tumor’s hormone receptors, either directly in the case of tamoxifen, an ER blocker, or indirectly in the case of aromatase inhibitors, which inhibit a key enzyme in estrogen’s biosynthetic pathway (Rothenberger, Somasundaram, & Stabile, 2018; Waks & Winer, 2019).

Compared to hormone therapy or even surgery and radiation therapy, chemotherapy is quite nonspecific. Chemotherapeutic agents interfere with various processes involved in cell proliferation, killing rapidly dividing cancer cells but also normal cells such as those that make up hair follicles or the gut epithelium, and immune cells (Zurrida & Veronesi, 2015). Although chemotherapy is famous for its sometimes brutal adverse side effects, it is the only systemic therapy in wide use that is effective against TNBC. Chemotherapeutic formulations include docetaxel/cyclophosphamide, doxorubicin/cyclophosphamide, and cyclophosphamide/methotrexate/5-fluorouracil for lower-risk patients, and doxorubicin/taxane treatments for higher risk patients (Waks & Winer, 2019). Anthracyclines, a class of chemotherapeutic drugs including doxorubicin (also known as adriamycin), will be discussed in greater detail below.
Finally, immunotherapy represents a newer approach to cancer treatment. Much research is currently being conducted to develop new immunotherapeutic drugs. As discussed above, a hallmark of cancer is its ability to evade immune detection. Current approaches have targeted immune-suppressive proteins produced by tumors as a means of re-sensitizing the immune system to tumoral antigens (Alsaab et al., 2017; Ernst & Anderson, 2015; Zugazagoitia et al., 2016). These strategies have met with some success and will surely see more in the coming years.

1.5. Cancer Treatment and Cell Death

Regardless of modality, the ultimate goal of cancer treatment is to remove and/or kill cancer cells. Putting aside surgical intervention, all other cancer treatments aim to cause cell death in some form, of which there are many.

1.5.1. Necrosis. Until the codification of apoptosis in 1972 (Kerr et al., 1972), necrosis was the only widely recognized form of cell death. In some ways, necrosis is the simplest form of cell death. Largely unregulated, it is generally caused by excessive stress to the cell in the form of injury, heat, lack of oxygen, or necrotizing infection. Necrosis is characterized by multiple breaches in the plasma membrane through which cellular contents can leak out (and through which other substances can potentially enter) (Lodish et al., 2016).

1.5.1.1. Necroptosis. Necroptosis, a variant of necrosis, is more structured. Necroptosis is activated when tumor necrosis factor alpha (TNFα) binds to TNFR1, its receptor; it can also be initiated by other “cell murder” ligand receptor pairs. Receptor binding causes receptor interacting protein 1 (RIP1) and 3 (RIP3) to form a complex called the necrosome. In the absence of caspase-8, the initiating caspase related to cell murder processes (discussed in more detail below), the necrosome complex phosphorylates mixed lineage kinase-like pseudokinase
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(MLKL). Phosphorylated MLKL executes necrosis by oligomerizing, translocating to the membrane, and forming necrotic pores (D’Arcy, 2019; Gong et al., 2019). Necroptosis can be either pro- or anti-cancer; the process can act as a fail-safe when caspase-8 is nonfunctional, but it can also cause inflammation that promotes cancer formation and growth (Gong et al., 2019).

1.5.2. **Apoptosis.** Apoptosis is the most famous form of programmed cell death. As mentioned above, cells undergoing apoptosis shrink and fragment, and the fragments are taken up by phagocytic cells for disposal. It is an endergonic, highly ordered process that can be triggered by a variety of stimuli including DNA damage, detachment from the ECM (also called anoikis), cell murder signals, or the absence of stimulus by the various trophic factors that cells require to stay alive, like the testosterone that prostate gland cells require (as in the earlier example) (Paoli, Giannoni, & Chiarugi, 2013). Once apoptosis is activated, it follows one of two branches of a prescribed signal cascade involving the cleavage of inactive procaspases to active caspases. Intrinsic signals—for example, DNA damage—cause the release of cytochrome $c$ from the mitochondrial intermembrane space. Cytochrome $c$ binds to and activates apoptotic protease activating factor 1 (APAF1). APAF1 cleaves procaspase-9 to the initiator caspase-9. Caspase-9 then cleaves procaspase-3 to the effector or executioner caspase-3, which causes a signal cascade that leads to cell death as described above (Lodish et al., 2016). Apoptosis triggered by an extrinsic signal—for example, binding of a cell murder ligand to its receptor—follows a slightly different pattern, activating the death inducing signaling complex (DISC). DISC cleaves procaspase-8 to the initiator caspase-8, which then cleaves procaspase-3 to caspase-3 in the same manner as caspase-9 (D’Arcy, 2019). On top of the stereotyped manner in which apoptosis proceeds, it is also regulated by a variety of other proteins, including members of the Bcl-2 and IAF families of proteins (Lodish et al., 2016).
1.5.3. Pyroptosis. Apoptosis is not the only form of programmed cell death regulated by caspase activation. Pyroptosis, inflammation-mediated cell death, is characterized by activation of caspase-1. Pyroptosis is often caused by a bacterial infection, especially in response to the bacterial protein flagellin (as well as other proteins of varied provenance). Caspase-1 activation causes pore formation in the plasma membrane, which allows pyoptotic components to leak into the extracellular space and stimulate pyroptosis in neighboring cells, causing a chain reaction of cell death. In fact, pyroptosis may be an attempt to control the spread of bacterial infection, but the cascade of cell death can also be maladaptive, especially in the setting of chronic sterile inflammation (Bergsbaken, Fink, & Cookson, 2009).

1.5.4. Autophagy. The last relevant iteration of programmed cell death is autophagy, literally “self-eating.” As a physiological event, cells utilize autophagy to recycle cell components for use in new macromolecules or to be broken down further for energy. Cells resort to autophagy when under stress, especially in starvation conditions when other materials are not available. Autophagy is initiated by the formation of a concave double membrane structure within the cytoplasm. The structure grows to engulf a portion of the cytosol, finally closing to form a vesicle, termed a “phagophore.” The phagophore is trafficked to the lysosome where the contents can be degraded and used for other purposes (Lodish et al., 2016). In more extreme cases, autophagy can result in the destruction of the cell; this often occurs to remove old cells from a larger population, but autophagy is used to destroy early cancer cells as well (D’Arcy, 2019). Malfunctions in the autophagy system can ablate the latter capacity, leading to the growth of cancers. In fact, beclin-1, a key component to the protein complex that promotes phagophore formation, is deleted in 40-75% of breast, ovarian, and prostate cancer cases (Mizushima,
Levine, Cuervo, & Klionsky, 2008; Poillet-Perez & White, 2019). This fact underscores how important it is to transforming cancer cells that they avoid death by autophagy.

1.6. Anthracyclines

Even before the many forms of programmed cell death had been described, clinicians had begun to administer drugs reliant on these processes. Anthracyclines, one such class of chemotherapeutic drugs, have been used widely since the 1960s for various forms of cancer and are still among the most effective chemotherapeutics (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004). The first anthracycline discovered, daunorubicin (DNR), was isolated in 1959 from *Streptomyces peucetius*, a soil bacterium collected in Italy (Di Marco, Cassinelli, & Arcamone, 1981). Later scientists applied mutagenic agents to *S. peucetius*, giving rise to a new subspecies that was able to produce a modified form of DNR that they called adriamycin but is better known today as doxorubicin (DOX) (Arcamone et al., 1969). Happily, the new drug turned out to be even more effective at killing cancer cells than the original (Volkova & Russell, 2011).

Anthracyclines induce cell death by interfering with the cell’s replication machinery. Specifically, they bind tightly to topoisomerase II, the enzyme responsible for relieving tension brought on by supercoiling during DNA synthesis. The interaction between the anthracycline, the enzyme, and the DNA strand forms a covalent bond between the double-stranded DNA and the anthracycline (Marinello, Delcuratolo, & Capranico, 2018). The resulting ternary complex halts the enzyme’s motion down the DNA strand, causing double-strand breaks and interfering with DNA synthesis (Binaschi et al., 2001).

As might be expected when administering a drug with such an aggressive mechanism, anthracyclines have major adverse side effects. Indeed, patients given anthracyclines to treat cancer exhibit the adverse reactions common to many chemotherapeutics: mucositis,
nausea/vomiting/diarrhea, alopecia, fatigue, etc. (Ansari et al., 2017). The most concerning side effect associated with anthracycline treatment, however, is cardiac and skeletal muscle toxicity. Cardiac damage manifests as congestive heart failure, fibrosis, hypertrophy (Lipshultz et al., 1991), and arrhythmias (Larsen et al., 1992). Damage is dose-dependent, beginning above a cumulative dose of 350 mg/m² body surface area (BSA) and increasing sharply above 550 mg/m² BSA (Volkova & Russell, 2011). Damage can occur acutely and subacutely, even years after treatment (Steinherz, Steinherz, Tan, Heller, & Murphy, 1991); one study found that 65% of patients studied had progressive cardiac anomalies 6 years or more post-treatment (Lipshultz et al., 1991).

There is some debate as to the exact etiology of the cardiotoxic effects of doxorubicin. The conjugated ring structure of anthracyclines allows for redox cycling with nearby molecules. As such, anthracyclines are highly prone to creating radicals in the form of reactive oxygen and nitrogen species, which can accumulate and cause indiscriminate damage to the cell (Cappetta et al., 2017). The radical formation hypothesis gains credence from the observation that mitochondria are especially susceptible to doxorubicin-mediated damage; doxorubicin has a high binding affinity for cardiolipin, which is concentrated in the inner mitochondrial membrane (Schlame, Rua, & Greenberg, 2000). However, other evidence argues for the position that anthracycline cardiotoxicity is due to interactions between anthracyclines and the topoisomerase IIβ (TopIIβ) isoform, which is prevalent in quiescent cells like (cardio-)myocytes. Anthracycline-TopIIβ interactions have been observed to cause double-strand DNA breaks and mitochondrial disturbances within these cells (McGowan et al., 2017). Experiments have demonstrated that cells depleted of TopIIβ exhibited reduced sensitivity to anthracycline treatment (Lyu et al., 2007); the same effect appeared in a TopIIβ-deficient mouse model (Zhang
et al., 2012). These results suggest that radical production may be a consequence, rather than a cause, of anthracycline toxicity.

Regardless of the exact source of cardiotoxicity, researchers have made various attempts at preventing it. Development of new molecules has been somewhat successful: epirubicin, a new anthracycline, is as effective as doxorubicin per milligram but can be delivered at twice the dose while maintaining the same level of toxicity (Robert, 1993). Likewise, encapsulating doxorubicin in pegylated liposomes reduces the risk of adverse reactions while maintaining efficacy against cancer (Ansari et al., 2017; Milla, Dosio, & Cattel, 2012). Nevertheless, cardiotoxicity of these alternative formulations is still relatively high.

Interestingly (given the previous discussion of radical-mediated damage), antioxidants have garnered interest in preventing damage from radicals. However, they have not seen success in clinical trials, perhaps because they target the wrong actor (Cappetta et al., 2017). Other chemicals have shown more promise: in preclinical studies, sildenafil and other phosphodiesterase 5 (PDE5) inhibitors have been shown to both increase DOX efficacy and decrease cardiotoxicity (A. Das et al., 2016, 2010), as has beetroot extract (S. Das, Filippone, Williams, Das, & Kukreja, 2016). Unfortunately, a recent clinical study testing the safety and efficacy of sildenafil in preventing DOX-mediated cardiotoxicity found that although sildenafil was safe, it had no effect on important indicators of cardiac function, at least at doses of DOX below 300 mg/m² (Poklepovic et al., 2018). Thus, the hunt for cardioprotective drugs continues.

1.7. Exosomes: A New Approach to Cardiotoxicity

The newest frontier in cardioprotection does not involve new chemical formulations but rather chemotherapeutic treatment in combination with exosome isolates. However, before
discussing the advantages of exosome treatment, I will first review the recent science regarding exosome formation and characteristics.

Exosomes are small extracellular vesicles (EVs) between roughly 50 and 150 nm in diameter (size ranges vary slightly among investigators). They can be differentiated from microvesicles, another type of small EV, by their expression of specific surface markers, namely the tetraspanins CD9, CD63, and CD81. Biologically speaking, exosomes are defined by the process by which they are formed. The precursors of mature exosomes form through invaginations of the early endosome membrane, which form small intraluminal vesicles (ILVs) within the endosomal lumen, now termed the multivesicular body (MVB). The exact mechanism by which ILVs are formed is not clear, although the endosomal sorting complex required for transport (ESCRT) is known to participate in most (though not all) cases. ILVs within the MVB can be released as exosomes via fusion of the MVB with the plasma membrane, which ejects the exosomes into the extracellular space. Alternatively, the MVB can fuse with the lysosome to recycle the ILVs and their contents (Hessvik & Llorente, 2018; Maas, Breakefield, & Weaver, 2017).

The first researchers to study exosomes hypothesized that the vesicles were used for cellular waste disposal based on the fact that maturing reticulocytes seemed to be dumping unnecessary surface proteins via exosome release (Johnstone, Mathew, Mason, & Teng, 1991). In the years since, EVs (including exosomes) have been found in every biological fluid that has been tested, and in vitro-grown cell lines have been found to release exosomes into their cell media (Hessvik & Llorente, 2018). Exosomes contain various macromolecules within their lumens, including functional mRNA (but no detectable rRNA) (Valadi et al., 2007) and microRNAs. Exosomes also carry segments of DNA, but their function is unknown (Lázaro-
Ibáñez et al., 2014). Because the populations of miRNAs found within exosomes differ from those within the mother cell, it is hypothesized that miRNAs are specifically trafficked to exosomes for export (Wang, Zhang, Weber, Baxter, & Galas, 2010). Exosomal membranes also contain proteins that serve various purposes, including assisting with docking to recipient cells (French, Antonyak, & Cerione, 2017).

Since the original reticulocyte studies, it has become clear that the main purpose of exosomes is cell-to-cell communication. In normal cells, exosomes are important to such diverse processes as the formation of the neuromuscular junction (Maggio et al., 2019), stem cell differentiation (Quesenberry, Aliotta, Deregibus, & Camussi, 2015), wound healing (Han, Tran, Chang, Azar, & Zieske, 2017; Quesenberry et al., 2015), and many others. Exosomes are important to cancer cell-to-cell communication as well (Al-Sowayan, Al-Shareeda, & Al-Hujaily, 2019), both within the tumor microenvironment (Wu, Zhou, Lv, Zhu, & Tang, 2019) and between tumors and distant metastases (Wortzel, Dror, Kenific, & Lyden, 2019). In addition to exosomes’ apparent importance in communication, they may also play a role in non-communicative transfer of macromolecules. Exosomes are capable of delivering free fatty acids to cardiac cells (Garcia et al., 2019), and they may even play a role in waste disposal after all via autophagy (Gudbergsson & Johnsen, 2019).

Finally, and most relevant to the current study, there is evidence that exosomes can play a role in cardioprotection. Exosomes derived from embryonic stem (ES) cells are capable of decreasing ischemia/reperfusion-mediated damage to the heart on a variety of axes (decreased infarct size, increased angiogenesis, reduced fibrosis and remodeling, improved contractility) (Davidson & Yellon, 2018). Furthermore, in vitro exosome treatment was shown to decrease DOX-mediated inflammation and pyroptosis in mouse soleus muscle (Tavakoli Dargani, Singla,
Johnson, Kukreja, & Singla (University of Central Florida, Orlando)\(^3\) showed that treatment of mice with DOX for 56 days significantly lowered grip strength. However, treatment with ES exosomes on alternating days surrounding DOX treatment significantly improved grip strength, rendering mice treated with ES exosomes indistinguishable from control mice in terms of grip strength. In addition, treatment with ES exosomes significantly attenuated muscle atrophy as well as pyroptosis caused by DOX treatment in mice. If the mechanism of anthracycline toxicity is truly the same in cardiac and skeletal muscle, exosome treatment should have similar mitigatory effects in cardiac tissue as well. Researchers have even used modified human umbilical cord stem cell-derived exosomes to treat infarct damage directly (Ni et al., 2019), while others have managed to load mesenchymal stem cell-derived exosomes with taxol, a chemotherapeutic drug unrelated to DOX or other anthracyclines, and deliver smaller doses of the drug with high antineoplastic efficacy (Melzer et al., 2019). Based on these achievements, the future for exosome-based treatment seems limitless.

1.8. The Present Study

The goal of the present study is to test the hypothesis that the addition of exosomes to doxorubicin treatment does not interfere with the chemotherapeutic effects of doxorubicin. We will measure the effects of combined exosome/doxorubicin treatment in several ways. We will examine the treatment’s effect on cell proliferation, cell death generally, and apoptosis specifically. We will also quantify any changes in levels of proteins important to initiation and prevention of apoptosis. Finally, we will attempt to determine the effects of exosome/doxorubicin treatment on EMT using a specific microRNA as a marker. We expect that the insights arising from the present study will contribute to a better understanding of the

\(^3\) The data referenced here was presented as part of a poster at the Experimental Biology 2019 conference (A. Das et al., 2019).
processes that underly previously observed interactions between exosomes, cancer, and cell death.
2. Materials and Methods

Cell Culture

Cancer cell lines MCF7 (ATCC HTB-22), MDA-MB-231 (ATCC HTB-26), and MDA-MB-468 (ATCC HTB-132) were purchased from ATCC. Cells were initially seeded in 25 cm² (T-25) flasks then transferred to 75 cm² (T-75) flasks via trypsinization once reaching 80% confluence. Cells were cultured at 37° C and 5% CO₂.

Cell Media and Reagents

All three cell lines were cultured in Dulbecco’s Modified Eagle Media (DMEM) with high glucose and pyruvate (Gibco) to which 10% Benchmark fetal bovine serum (FBS, Gemini Bio-Products) and 1% penicillin/streptomycin (10000 U/mL, Gibco) were added. Phosphate-buffered saline (PBS, pH 7.4, 1X) was obtained from Gibco.

Doxorubicin hydrochloride was obtained from Sigma and diluted to 1 μM. Exosomes isolated from human embryonic stem (ES) cells and mouse embryonic fibroblasts (MEF) at a concentration of 5 μg/mL were graciously provided by our collaborator Dr. Dinender Singla at the Burnett School of Medical Sciences at the University of Central Florida.

Microscopy

Cells were visualized using a Nikon Eclipse Ti-S phase contrast microscope. Digital photographs were obtained using an attached Nikon DS-Fi1 camera controlled by a Nikon DS-U3 camera controller and analyzed using the NIS Elements AR software (Nikon, version 3.2).

Doxorubicin/Exosome Treatment

Cells were allowed to grow to 60-80% confluence. Culture medium was removed, adherent cells were washed with PBS, which was then discarded. New media was added containing: no additives (control condition); 1 μM doxorubicin hydrochloride (“DOX”
condition); 10 μg/mL ES exosomes (“ES” condition); 10 μg/mL MEF exosomes (“MEF” condition); 1 μM doxorubicin hydrochloride and 10 μg/mL ES exosomes (“DOX+ES” condition); or 1 μM doxorubicin hydrochloride and 10 μg/mL MEF exosomes (“MEF” condition). Cells were incubated in treatment media at 37°C and 5% CO₂ for 48 hours.

**Cell Death Assay**

After doxorubicin/exosome treatment, culture medium was aspirated from the growth flask or plate. Adherent cells were washed with PBS, which was then aspirated and added to the vessel containing the previously-collected media. Trypsin-EDTA (0.25%, Gibco) was added to the flask or plate and incubated for five minutes at 37°C and 5% CO₂ to detach the cells from the surface. A portion of the previously-collected media/PBS was added to the cell-trypsin suspension to inactivate the trypsin. The suspension was aspirated and added back to the vessel containing the remaining media/PBS mixture. The vessel was centrifuged at 3,000 rcf for 5 minutes to pellet the cells out of solution. The liquid phase was poured off and the pellet was resuspended in 1 mL of cold PBS. 100 μL of the suspension was removed to a 1.5 mL Eppendorf tube to which was added 15 μL of trypan blue solution (0.4%, Sigma). 20 μL of the suspension was pipetted onto a Bright-Line Hemacytometer (Hausser Scientific) and cells in each quadrant were counted manually using a phase contrast microscope.

**Cell Proliferation Assay**

Cells were plated on 96-well plates and allowed to grow to 60-80% confluence. Cells were treated with doxorubicin hydrochloride/exosomes as described above. After the 48 hour treatment period, media was removed and 110 μL of a 1:5 mixture of CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) MTS reagent and culture medium was added to each well. Plates were incubated for 30 minutes at 37°C and 5% CO₂. After incubation,
absorbance at 490 nm was recorded using a VERSA Max tunable microplate reader and the SoftMax Pro software (version 5).

**Flow Cytometry**

Cells for flow cytometry were plated in six-well plates. After reaching 80% confluency, cells were treated with doxorubicin hydrochloride and exosomes, as described above, with two wells per treatment condition. After 48 hours of treatment, cells were harvested via trypsinization as described above, keeping harvested cells as close to 0° C as possible. Samples were prepared from the harvested cells using the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 & Propidium Iodide (Invitrogen) according to the manufacturer’s instructions. Samples were analyzed using a BD FACSCanto II Analyzer in conjunction with the FACSDIVA analysis software.

**Protein Isolation/Quantification**

Protein was isolated from pelleted cells post-treatment using Cell Lysis Buffer (10X, Cell Signaling Technology) with Protease/Phosphatase Inhibitor Cocktail (100X, Cell Signaling Technology) according to the manufacturer’s instructions. Isolated protein was quantified via Bradford assay using a SmartSpec 3000 spectrophotometer (Bio-Rad) and Quick Start™ Bradford 1x Dye Reagent (Bio-Rad) in a 500:1 reagent-to-protein dilution.

**SDS PAGE**

Protein samples were mixed with Laemmli sample buffer (Bio-Rad) in a 1:1 ratio and boiled at 100° C for 5 minutes to denature the proteins. The lysates were then loaded in precast 4–20% Criterion TGX Protein Gel (Bio-Rad) immersed in Tris/Glycine/SDS Buffer (Bio-Rad) prepared according to the manufacturer’s instructions. A Dual Color, 10-250 kD Precision Plus Protein Standard (Bio-Rad) was used to measure protein migration. Gel electrophoresis was
performed using a 200 V current provided by a PowerPac Basic power supply (Bio-Rad) for approximately 50 minutes.

**Western Blot**

Separated proteins were transferred from the gel to a nitrocellulose membrane (2 μm, Bio-Rad) immersed in Tris/Glycine Buffer (Bio-Rad), prepared according to the manufacturer’s instructions. Transfer was accomplished via a 300 mA current over one hour. The membrane was stained using Ponceau S solution (Sigma-Aldrich) to confirm protein transfer. The membrane was washed to remove Ponceau stain with 0.1% TWEEN 20 (Sigma) in Tris-buffered saline (TBS, Bio-Rad), prepared according to the manufacturer’s instructions. The membrane was developed according to a general Western blot protocol from Abcam. The membrane was incubated with antibodies for Bax (D2E11, Cell Signaling Technologies), Bcl-2 (D55G8, Cell Signaling Technologies), and GAPDH (14C10, Cell Signaling Technologies) in a 5% w/v bovine serum albumin (BSA, Sigma), 0.1% TWEEN 20 TBS solution at a dilution recommended by the manufacturer for each antibody. The membrane was further incubated in anti-mouse or anti-rabbit IgG, horse radish peroxidase (HRP)-linked antibodies (Cell Signaling Technologies) appropriate to the given primary antibody as per the manufacturer’s instructions.

Blots were visualized using one of two methods. One portion was visualized using Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer) as a chemiluminescent reagent to expose radiographic film (BioExcell autoradiographic film, WorldWide Medical Products). The exposed film was developed using a Kodak X-OMAT 2000A processor. The other portion of blots was visualized using Clarity Western ECL Substrate (Bio-Rad) and a ChemiDoc XRS+ imaging system (Bio-Rad). The images were processed with
the ImageLab software (Bio-Rad). Blot images were analyzed using ImageJ (Rueden et al., 2017).

**RNA Isolation**

Treated cells were collected via trypsinization and flash-frozen in liquid nitrogen. Total RNA, including small RNA, was isolated from the samples using the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The concentration and purity of the isolated RNA was measured using a NanoDrop One microvolume UV-Vis spectrophotometer (Thermo Scientific).

**cDNA Library Preparation and RT-PCR**

CDNA of micro-RNA (miRNA) present in the isolated RNA was synthesized using the microRT reverse transcription kit (Applied Biosystems). 10 ng of total RNA was subjected to reverse complement strand synthesis with microRNA-specific stem-loop primers according to the manufacturer’s instructions. Reverse transcription polymerase chain reaction was conducted on a CFX96-C1000 Touch Real-Time PCR Detection System (Bio-Rad) using the following conditions: 16° C for 30 minutes; 42° C for 30 minutes; and 85° C for 5 minutes.

Expression of miR-200c was quantified via real-time PCR (RT-PCR) using amplicon-specific Taqman assay probes and primers (Applied Biosystems) using a CFX96-C1000 Touch Real-Time PCR Detection System. Conditions for the RT-PCR run were as follows: 50° C for 2 minutes; 95° C for 3 seconds; 60° C for 39 cycles of 1 minute per cycle. The small nucleolar RNA sno-202, a housekeeping snoRNA, was used to normalize miRNA expression.

MiRNA relative quantification data obtained from RT-PCR was analyzed using the $2^{-\Delta\Delta CT}$ method and normalized to the sno-202 control.
Data Analysis

Data are reported as mean or mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey’s multiple comparisons test was performed using GraphPad Prism version 8.1.2 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.
3. Results

In the present study, we used three cancer cell-lines for studying the effect of embryonic stem (ES) cell-derived exosomes on doxorubicin hydrochloride (DOX)-induced cell killing. These cell lines were MCF7, MDA-MB-231 and MDA-MB-468. MCF7 are ER- and PGR-positive but HER2-negative and are a model for hormone therapy. MDA-MB-231 and MDA-MB-468 are triple negative and are a model for chemotherapy. Exosomes were administered at a concentration of 10 μg/mL based on the results of cell viability assays comparing the antiproliferative effects of 5 μg/mL, 10 μg/mL, and 20 μg/mL concentrations (below, Figures 6a-c). The assays showed no significant difference between the three concentrations in terms of antiproliferative effects, on cells from either the MCF7 or MDA-MB-231 cell lines. Although a higher dose of exosomes was associated with a trend of increased antiproliferative activity in the MCF7 cells, the MDA-MB-231 cells responded to an increased dose of exosomes with a U-shaped trend, where the 10 μg/mL concentration exhibited the largest antiproliferative effect. As such, the 10 μg/mL concentration was chosen for this study.

3.1. Cell Death Assay with Trypan Blue

**Exosome/DOX treatment increases overall cell death.** Treatment of MCF7 cells with DOX/ES exosomes for 48 hours significantly increased cell death (31.9 ± 1.3% cell death, n=4) compared to control (6.2 ± 1.1% cell death, n=4, p<0.0001) and compared to DOX alone (22.9 ± 0.3% cell death, n=4, p=0.002). Cell death was also significantly increased after treatment with DOX/MEF for 48 hours (15.2 ± 2.4% cell death, n=4) compared to control (p=0.002) (Figure 1a).

Treatment of MDA-MB-231 cells with DOX/ES exosomes for 48 hours significantly increased cell death (49.2 ± 2.0% cell death, n=4) compared to control (11.4 ± 0.01% cell death,
n=4, p<0.0001) and compared to DOX alone (38.2 ± 1.2% cell death, n=4, p<0.0001). Cell death was also significantly increased after treatment with DOX/MEF for 48 hours (42.2 ± 0.5% cell death, n=4) compared to control (p<0.0001) but was not significantly different from DOX alone (p=0.13) (Figure 1b).

### 3.2. Flow Cytometry with Annexin V/Propidium Iodide

**Exosome/DOX treatment increased overall cell death.** Treatment of MCF7 cells with DOX/ES for 48 hours decreased the number of viable cells and therefore increased the number of dead cells: 72.0% viable cells versus 85.1% viable cells under the control condition and 75.9% viable cells under DOX-only treatment. Treatment with DOX/MEF also decreased the number of viable cells (69.8% viable cells). We counted 10,000 cells for each experimental condition (Figure 2).

Treatment of MDA-MB-231 cells with DOX/ES for 48 hours decreased the number of viable cells: 44.2% viable cells (of 3,807 cells counted) versus 91.4% viable cells under the control condition (of 6,102 cells counted) and 53.5% viable cells under DOX-only treatment (of 3,660 cells counted). Treatment with DOX/MEF also decreased the number of viable cells (45.5% viable cells of 4,067 cells counted) (Figure 3).

Treatment of MDA-MB-468 cells with DOX/ES for 48 hours decreased the number of viable cells: 71.6% viable cells versus 95.3% viable cells under the control condition and 80.9% viable cells under DOX-only treatment. Treatment with DOX/MEF also decreased the number of viable cells (57.7% viable cells). We counted 10,000 cells for each experimental condition (Figure 4).

**Exosome/DOX treatment increased apoptosis.** Treatment of MCF7 cells with DOX/ES for 48 hours increased early apoptosis, as measured by the percentage of cells with coordinates in
quadrant 4: 11.2% versus 0.5% under the control condition and 9.0% when treated with DOX only. Treatment with DOX/MEF also increased early apoptosis to 11.2%. Note that the number of cells counted are reported in the previous section (Figure 2).

Treatment of MDA-MB-231 cells with DOX/ES for 48 hours increased early apoptosis, as measured by the percentage of cells with coordinates in quadrant 4: 40.1% versus 6.4% under the control condition and 31.1% when treated with DOX only. Treatment with DOX/MEF also increased early apoptosis to 41.0% (Figure 3).

Treatment of MDA-MB-468 cells with DOX/ES for 48 hours increased early apoptosis, as measured by the percentage of cells with coordinates in quadrant 4: 22.1% versus 2.6% under the control condition and 13.7% when treated with DOX only. Treatment with DOX/MEF also increased early apoptosis to 35.9% (Figure 4).

3.3. MTS Cell Proliferation Assay

**Exosome/DOX treatment increases or maintains DOX antiproliferative effects.**

Proliferation of MCF7 cells treated with DOX/ES exosomes for 48 hours (43.2 ± 1.2% viable cells, n=6) was significantly lower than control (100.0 ± 3.3% viable cells, n=10, p<0.0001) and DOX alone (62.2 ± 4.4% viable cells, n=12, p=0.006). Proliferation of MCF7 cells treated with DOX/MEF exosomes (31.8 ± 3.3% viable cells, n=6) was significantly lower than control (p<0.0001) and DOX alone (p<0.0001) (Figure 6a).

Proliferation of MDA-MB-231 cells treated with DOX/ES exosomes for 48 hours (25.6 ± 0.7% viable cells, n=6) was significantly lower than control (100.0 ± 2.8% viable cells, n=10, p<0.0001) but did not differ from cells treated with DOX alone (25.8 ± 0.5% viable cells, n=12, p>0.99). Proliferation of MDA-MB-231 cells treated with DOX/MEF exosomes (23.4 ± 0.9%
viable cells, \( n=6 \) was significantly lower than control \( (p<0.0001) \) but did not differ from cells treated with DOX alone \( (p>0.99) \) (Figure 6b).

Proliferation of MDA-MB-468 cells treated with DOX/ES exosomes for 48 hours \( (60.1 \pm 1.7\% \text{ viable cells, } n=16) \) was significantly lower than control \( (100.0 \pm 5.0\% \text{ viable cells, } n=14, \ p<0.0001) \) but did not differ from cells treated with DOX alone \( (53.3 \pm 1.6\% \text{ viable cells, } n=14, \ p=0.96) \). Proliferation of MDA-MB-468 cells treated with DOX/MEF exosomes \( (53.1 \pm 1.3\% \text{ viable cells, } n=16) \) was significantly lower than control \( (p<0.0001) \) but did not differ from cells treated with DOX alone \( (p>0.99) \) (Figure 6c).

3.4. Real-Time Polymerase Chain Reaction

Treatment with DOX/Exosomes Decreases expression of miR-200c. Treatment of MCF7 cells with DOX/ES exosomes for 48 hours decreased expression of miR-200c, a 0.77-fold change compared to control \( (\pm 0.05, n=3, p=0.36) \). Expression was also decreased compared to DOX alone \( (1.02 \pm 0.05\text{-fold change compared to control}; \ p=0.24) \). Treatment with both ES exosomes alone and with DOX/MEF exosomes produced significant changes compared to both control \( (\text{ES: } 0.65 \pm 0.07\text{-fold change, } p=0.033; \ \text{DOX/MEF: } 0.63 \pm 0.05\text{-fold change, } p=0.02) \) and DOX alone \( (\text{ES: } p=0.02; \ \text{DOX/MEF: } p=0.01) \) (Figure 7a).

Treatment of MDA-MB-231 cells with DOX/ES exosomes for 48 hours did not decrease expression of miR-200c, a 0.89-fold change compared to control \( (\pm 0.03, n=3, p=0.89) \), but likely did decrease expression compared to DOX alone \( (1.13 \pm 0.07\text{-fold change compared to control}; \ p=0.29) \). Treatment with ES exosomes alone also may have decreased expression compared to control \( (0.81 \pm 0.11\text{-fold change, } p=0.48) \) and likely decreased expression compared to DOX alone \( (p=0.09) \) (Figure 7b).
Treatment of MDA-MB-468 cells with DOX/ES exosomes for 48 hours significantly decreased expression of miR-200c, a 0.55-fold change compared to control (± 0.05, n=3, p=0.01). Expression may have also decreased compared to DOX alone (0.71 ± 0.7-fold change compared to control; p=0.66) (Figure 7c).

3.5. Western Blot

**Exosome/doxorubicin treatment increased Bax expression.** Treatment of MCF7 cells with a combination of doxorubicin hydrochloride (DOX) and embryonic stem cell-derived (ES) exosome solution (DOX/ES) for 48 hours caused an appreciable increase in expression of Bax (mean relative density: 1.51, n=2) compared to control (mean relative density: 1.00) and a possible increase compared to treatment with DOX alone (mean relative density: 1.39).

Treatment with mouse embryonic fibroblast-derived (MEF) exosomes in combination with DOX (DOX/MEF) also caused an appreciable increase in Bax expression (mean relative density: 1.59, n=2) compared to control and a noticeable increase compared to treatment with DOX alone (Figure 8a).

Treatment of MDA-MB-231 cells with DOX+ES under the same conditions also appeared to increase the expression of Bax (mean relative density: 1.35, n=2) compared to control (mean relative density: 1.02) (Figure 9a).

**Exosome/doxorubicin treatment decreased Bcl-2 expression.** Treatment of MCF7 cells with DOX and ES exosomes as above caused a slight decrease in the expression of Bcl-2 (mean relative density: 0.83, n=2) compared to control (mean relative density: 1.00) and treatment with DOX alone (mean relative density: 0.98) (Figure 8b).
Treatment of MDA-MB-468 cells with DOX/ES exosomes caused a decrease in the expression of Bcl-2 (mean relative density: 0.73, n=2) compared to control (mean relative density: 1.00); there was no change compared to DOX alone (mean relative density: 0.71). Treatment with DOX+MEF also caused a decrease in Bcl-2 (mean relative density: 0.75, n=2) compared to control but likely no change compared to treatment with DOX alone (Figure 10b).

**Exosome/doxorubicin treatment increased expression of Bax compared to Bcl-2.**

Treatment of MCF7 cells with DOX/ES increased the expression of Bax compared to Bcl-2 (when expressed as a ratio of Bax/Bcl-2; mean relative density: 1.82) versus control (mean relative density: 1.00). Expression of Bax versus Bcl-2 was also noticeably increased compared to DOX alone (mean relative density: 1.44). Treatment with MEF/DOX also increased the expression of Bax versus Bcl-2 (1.76) compared to control and was increased compared to treatment with DOX alone (Figure 8c).

Treatment of MDA-MB-231 cells with ES/DOX increased expression of Bax versus Bcl-2 (mean relative density: 1.40, n=2) compared to control (mean relative density: 1.00). Expression was not changed compared to treatment with DOX alone (mean relative density: 1.39) (Figure 9c).

Treatment of MDA-MB-468 cells with ES/DOX did not clearly increase expression of Bax versus Bcl-2 (mean relative density: 1.07, n=2) compared to control (mean relative density: 1.00) nor when compared to DOX alone (mean relative density: 1.22). Likewise, treatment with MEF/DOX did not clearly increase the expression of Bax versus Bcl-2 (mean relative density: 1.36) compared to control or compared to treatment with DOX alone (Figure 10c).

**Treatment with exosomes alone did not change measured protein expression.**

Treatment of MCF7 cells for 48 hours with ES exosomes alone did not change Bax expression
compared to control (mean relative density: 1.05 vs. 1.00, n=2) or Bcl-2 expression compared to control (mean relative density: 1.13 vs. 1.00, n=2) (Figure 8a). Likewise, treatment with MEF exosomes alone did not change Bax expression compared to control (mean relative density: 1.09 vs. 1.00, n=2) or Bcl-2 expression compared to control (mean relative density: 1.07 vs. 1.00, n=2) (Figure 8b). The ratio of Bax to Bcl-2 expression was not altered by treatment with ES alone compared to control (mean relative density: 0.93 vs. 1.00, n=2) or by treatment with MEF alone compared to control (mean relative density: 1.03 vs. 1.00, n=2) (Figure 8c).

Treatment of MDA-MB-231 cells for 48 hours with ES exosomes alone did not change Bax expression compared to control (mean relative density: 1.08 vs. 1.02, n=2) or Bcl-2 expression compared to control (mean relative density: 1.07 vs. 1.01, n=2) (Figure 9a). Treatment with MEF exosomes alone also did not change Bax expression compared to control (mean relative density: 0.93 vs. 1.02) or Bcl-2 expression compared to control (mean relative density: 1.01 vs. 1.01, n=2) (Figure 9b). The ratio of Bax to Bcl-2 expression was not altered by treatment with ES alone compared to control (mean relative density: 0.99 vs. 1.00, n=2) or by treatment with MEF alone compared to control (mean relative density: 0.92 vs. 1.00, n=2) (Figure 9c).

Treatment of MDA-MB-468 cells for 48 hours with ES exosomes alone did not increase and may have decreased Bax expression compared to control (mean relative density: 0.77 vs. 1.00, n=2) (Figure 10a) and did not change Bcl-2 expression compared to control (mean relative density: 0.98 vs. 1.00, n=2) (Figure 10b). Treatment with MEF exosomes alone also did not increase and may have decreased Bax expression compared to control (mean relative density: 0.81 vs. 1.00, p=0.049) and did not change Bcl-2 expression compared to control (mean relative density: 1.03 vs. 1.00, n=2). The ratio of Bax to Bcl-2 expression was not increased and may
have been decreased by treatment with ES alone compared to control (mean relative density: 0.79 vs. 1.00, n=2) and by treatment with MEF alone compared to control (mean relative density: 0.79 vs. 1.00, n=2) (Figure 10c).
4. Discussion

Doxorubicin (DOX) is one of the most widely used chemotherapeutic drugs. It is very effective at treating a wide range of cancers, in particular breast and esophageal cancers, osteosarcoma, Kaposi’s sarcoma, soft tissue sarcomas, and both Hodgkin’s and non-Hodgkin’s lymphomas (Singal & Iliskovic, 1998). However, the use of DOX in cancer treatment is limited by its dose-dependent cardiotoxicity. As noted in the introduction, a cumulative dose of 350 mg/m² of body surface area (BSA) causes cardiotoxic effects to begin to quickly increase; above 500 mg/m² BSA, the risk of adverse effects on the heart increases to unacceptable levels. High concentrations of DOX carry a high risk of congestive heart failure (CHF), dilated cardiomyopathy, and death (Singal, Deally, & Weinberg, 1987; Singal & Iliskovic, 1998). Because there is no effective treatment for DOX-induced cardiotoxicity, patients treated with high doses of DOX have a poor prognosis. DOX is also harmful to skeletal muscle, causing muscle fatigue and weakness. However, recent data from our lab has produced encouraging results, showing a cytoprotective effect of embryonic stem cell-derived (ES) exosomes on DOX-induced inflammation and pyroptosis in Sol-8 slow-twitch soleus muscle cells (Tavakoli Dargani et al., 2018). Furthermore, unpublished data from our collaborator Dr. Dinender K Singla (University of Central Florida) showed improved muscle grip strength in mice following alternating treatments with DOX and ES exosomes. Although these data suggest that ES exosomes may have a significant cytoprotective effect, the effect of ES exosomes on cancer cells is not known. Therefore, the present study sought to investigate whether the addition of ES exosomes contributed to the killing of cancer cells by DOX treatment.

Our results showed that DOX/ES exosome treatment of cancer cells either increased or maintained the cell killing and antiproliferative effects of DOX alone. Western blot analysis
showed a marked increase in Bax compared to Bcl-2 in cells treated with a combination of ES or mouse embryonic fibroblast (MEF) exosomes and DOX compared to control across all three cell lines studied, and compared to DOX alone for the MCF7 cell line. These results suggest that DOX/ES exosomes treatment increases apoptosis across these cell lines.

As discussed in the introduction, cell death comes in many forms; the most relevant to the present study is apoptosis. Apoptosis can result from either intrinsic or extrinsic signals. Intrinsically mediated apoptosis occurs when cytochrome $c$ is released from the mitochondrial intermembrane space and activates APAF-1, causing a signaling cascade that results in cell death. Many signals can liberate cytochrome $c$; one such signal is the activation of the pro-apoptotic protein Bax, which forms oligomers that insert into the mitochondrial outer membrane and form a pore often referred to as the mitochondrial permeability transition pore (MPTP). Bcl-2, an anti-apoptotic protein, maintains the mitochondrial outer membrane's low permeability by binding to and sequestering Bax (Lodish et al., 2016, p. 1018). Thus, disruption of the Bcl-2/Bax interaction can precipitate apoptosis, as can overexpression of Bax or knockdown or deletion of $bcl-2$.

To measure apoptosis, we employed several experimental techniques. First, we performed cell death assays with trypan blue, a type of dye exclusion assay. This assay does not measure apoptosis directly; instead, it measures total cell death, which combines necrosis, apoptosis, and any other forms of cell death. The assay takes advantage of a property of all dead cells, namely the loss of membrane integrity that occurs upon death. Living cells tightly regulate their membrane permeability. Only very specific materials are allowed to pass across the membrane into the cytoplasm. When a cell dies, however, the mechanisms that govern this strict control unravel, and the membrane develops holes. Trypan blue, a water-soluble blue dye, can
pass through these holes and into the cell, staining it a deep blue color that can be visualized under a microscope (Strober, 2001). The number of living and dead cells can then be manually counted and the total amount of cell death can be obtained. In accordance with the protein expression data discussed above, cell death increased significantly in cells treated with DOX/ES exosomes compared to control as well as compared to DOX alone across both the MCF7 and MDA-MB-231 cell lines (Figures 1a and b). Again, the assay results encompass all kinds of cell death and not solely apoptotic cell death. Even so, the results should be consistent with other measures of cell death or apoptosis, assuming that other forms of cell death besides apoptosis are not a large source of variability within the bounds of the DOX/ES exosomes treatment paradigm.

Like the cell death assays discussed above, cell proliferation assays also indicated that treatment with DOX/ES exosomes contributed to the larger cell killing effect compared to DOX alone. The cell proliferation assays performed as part of this study differ from cell death assays in that they measure cell metabolism, not cell death. To assay for cell proliferation, cells were grown in a 96-well plate. Once the cells reach appropriate confluence, an MTS solution was added to the cell media and the cells were incubated for a short time. During incubation, healthy cells broke down the MTS substrate, forming a formazan dye that caused the cell media to change color in a manner proportionate to the amount of dye formed. The resulting color change was then measured using a microplate reader.

Like the cell death assays, the cell proliferation assays showed a decrease in cell activity for cells treated with DOX/ES exosomes compared to control across the MCF7, MDA-MB-231, MDA-MB-468 cell lines (Figures 6a-c). However, only the MCF7 cells showed a different response to the DOX/ES exosomes treatment and DOX treatment alone. The cell death assays, as

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4 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
well as the flow cytometry experiments which will be discussed below, showed increased
efficacy of the DOX/ES exosomes treatment over DOX alone for the MCF7, MDA-MB-231, and
(in the case of the flow cytometry data) MDA-MB-468 cell lines. Especially because of the large
difference in DOX growth suppression between the MCF7 and MDA-MB-231 cell lines in the
cell proliferation data (43% viable MCF7 cells versus 26% viable MDA-MB-231 cells under the
DOX/ES exosome condition), future experiments will be performed to validate the observation
that MDA-MB-231 proliferation is equally suppressed by treatment with DOX/ES exosomes and
DOX alone. What is more puzzling is the fact the DOX/ES exosomes and DOX/MEF exosomes
treatments had the same effect on cell proliferation for all three cell lines. In this case, the cell
proliferation data agreed with both the flow cytometry and protein expression data as discussed
above, neither of which showed a distinct difference between the DOX/ES and DOX/MEF
exosomes treatments. The similarity in effects of ES and MEF exosomes across experiments will
be discussed in depth below; suffice it to say until that discussion that the differences between
the new exosome populations needs to be characterized more fully.

As mentioned above, our results from the flow cytometry experiments showed increased
cell death, similar to the results of the cell death assays. However, unlike a simple dye exclusion
assay, flow cytometry can separate cells by type of cell death—here, necrosis versus apoptosis—
as well as a host of other characteristics.

To assess cell necrosis or apoptosis using flow cytometry, two dyes were added to the
cell suspension, one that binds to all dead cells and another that binds only to apoptotic cells. The
first, propidium iodide (PI), is a fluorescent dye that binds tightly to nucleotides by intercalating
between bases. As explained in the introduction, during later stages of cell death, the nuclear
envelope disintegrates and genetic material is broken into fragments. In addition, cell death
causes pores to open in the cell membrane, allowing the PI dye to enter the cell and bind to the nucleic acid fragments, much the same way that trypan blue enters dead cells during a dye exclusion assay (Nicoletti, Migliorati, Pagliacci, Grignani, & Riccardi, 1991; Riccardi & Nicoletti, 2006). Because PI is fluorescent, the amount of the dye in each cell can be quantitated individually by a flow cytometer and each measurement can be graphed along an axis of healthy to necrotic cells.

Although later stages of apoptosis can disrupt the integrity of the cell membrane, cells in the early stages of apoptosis have intact cell membranes. However, as the apoptotic process progresses, the membrane begins to change and phosphatidyl serine (PS), a membrane lipid, translocates from the inner leaflet to the outer leaflet (Vermes, Haanen, Steffens-Nakken, & Reutellingsperger, 1995). To measure the extent of PS translocation, annexin V, an antibody specific for PS, is added to the cell suspension. For the flow cytometry experiments, the annexin V antibodies are conjugated with a fluorescent protein that fluoresces at a different wavelength than PI, allowing signals from PI and annexin V to be separated from one another. Like the data obtained from PI staining, the annexin V data can be graphed along an axis from healthy cells to apoptotic cells. When the data from both dyes is combined into a single graph, cells with low annexin V and high PI signals can be assumed to be necrotic (and fall into quadrant 1); cells with high annexin V and low PI signals can be assumed to be in the early stages of apoptosis (and fall into Q4); and cells with high fluorescence from both dyes can be assumed to be in the late stages of necrosis.

The flow cytometry data showed that overall cell death increased due to DOX/ES exosomes treatment (Figures 2-4), which was congruent to the results of the cell death (dye exclusion) assays. Furthermore, cell death increased specifically as a result of increased
apoptosis: more cells showed signs of apoptosis after treatment with DOX/ES exosomes than control populations or those treated with DOX alone. These results suggest that the addition of exosomes did not increase necrosis directly. Indeed, there has not been any evidence to show that exosome treatment should increase necroptosis. These experiments did not make a distinction between necrosis and pyroptosis or autophagy. However, the large increase in apoptosis suggests that inflammation could be playing an important role in ES exosomes-induced increases in the cell-killing power of DOX. Further studies are needed to fully understand the mechanisms of early increase in apoptosis following treatment with DOX plus ES exosomes.

In addition to studying the effects of exosome treatment on cell proliferation and death, we also examined the role of exosomes in altering the expression of regulatory RNAs in cancer cells. Specifically, we looked at the expression of microRNAs (miRNAs), small (~22 nucleotide) strands of RNA that regulate protein translation, among other processes. To understand the significance of miRNAs to the cell, it is first important to understand their biogenesis. A segment of DNA that encodes a specific miRNA is transcribed to a pri-miRNA, a piece of RNA that ranges in length from hundreds to thousands of nucleotides (nt). The pri-miRNA remains in the nucleus, where an RNase III-family nuclease called Drosha cleaves it to a ~70 nt precursor miRNA (pre-miRNAs). The pre-miRNA assumes a hairpin configuration because of the palindromic sequence of the nucleotides. The pre-miRNA is then exported out of the nucleus to the cytoplasm via the RanGTP/exportin 5 system. In the cytoplasm, the pre-miRNA is processed into the mature ~22 nt miRNA by an enzyme called Dicer (Denli, Tops, Plasterk, Ketting, & Hannon, 2004). Finally, the mature miRNA is activated when it is loaded onto the Argonaute (Ago) binding protein, which forms the RNA-induced silencing complex (RISC). The complete RISC can then bind to target mRNA. Depending on the extent of the homology between the
miRNA sequence and the target, the bound miRNA can either inhibit translation, knocking down levels of the resulting protein, or lead to outright mRNA cleavage and destruction (Guo & Wang, 2019).

In the current study, we looked specifically at miRNAs belonging to the miR-200 cluster. This family includes miR-200a, -200b, -200c, miR-141, and miR-429 (Gregory et al., 2008). The miR-200 family targets ZEB1 and ZEB2 (zinc finger E box-binding homeobox 1 and 2), genes that code for transcription factors which negatively regulate the E-cadherin promoter (Korpal, Lee, Hu, & Kang, 2008). The interactions between the miR-200 family, ZEB1/2, and E-cadherin are heavily implicated in the epithelial-mesenchymal transition (EMT) process: cellular levels of E-cadherin play a large role in the initiation of EMT, which can contribute to many processes including embryonic development, wound healing, and cancer metastasis.

Of the members of the miR-200 family, our results showed that miR-200c was most affected by treatment with DOX/ES exosomes (Figures 7a-c). Although all members of the miR-200 family interact with ZEB1/2, each has its own nuances. MiR-200c targets transcripts for both ZEB1 and ZEB2 (Gregory et al., 2008). In turn, ZEB1 is capable of negatively regulating not only E-cadherin but also miR-200c. As such, the observed decrease in miR-200c could potentially lead to a feed-forward effect whereby expression of ZEB1/2, free from miR-200c repression, increases. The increase in ZEB1 could then cause a decrease in E-cadherin; at the same time, increased ZEB1 could also cause a further decrease in transcription of miR-200c (Berx & van Roy, 2009). Therefore, a decrease in miR-200c should lead to an increase in EMT.

However, our results showing a decrease of miR-200c following treatment with DOX/ES exosomes treatment is not consistent with the other observations. First, EMT is normally associated with later-stage, deadlier cancers (Otsuki, Saya, & Arima, 2018). EMT—
specifically that caused by ZEB1 expression—is associated with chemotherapeutic drug resistance in pancreatic cancer (Arumugam et al., 2009), and EMT is associated with worse clinical outcomes in breast cancer (Teschendorff, Journée, Absil, Sepulchre, & Caldas, 2007). Furthermore, studies of murine mammary cell lines (Robson, Khaled, Abell, & Watson, 2006) and cancer cells (Siegel & Massagué, 2003; Thiery, 2002) associated EMT with resistance to apoptosis. However, results from the cell growth/death experiments showed an increase in apoptosis (and therefore a decrease in chemotherapeutic drug resistance) as a result of DOX/ES exosomes treatment, in contrast to previously published work. As such, more work is needed to verify the observed increase in miR-200c after DOX/ES exosomes treatment and to characterize the effect of miR-200c on EMT. Also, further studies are required to investigate whether ZEB1 was increased in parallel to the decrease in miR-200c, through quantification of either the ZEB1 protein via Western blot or ZEB1 mRNA via real time PCR (RT-PCR). E-cadherin levels should also be interrogated directly, again either via Western blot of the protein or RT-PCR of the mRNA.

The other unexpected finding of this study was the lack of any clear difference in cell-killing efficacy of ES and MEF exosomes. In fact, some of our results showed that the MEF exosomes were more effective than the ES exosomes at stunting cell growth or inducing apoptosis (Figures 2, 4, 6a, 6c). It was expected that the human embryonic stem cell-derived exosomes would be effective against three human cancer cell lines under study while the MEF exosomes would not, due to differences in molecular composition as well as differences in species of origin, i.e. human versus mouse. Further extensive characterization of the exosomes derived from ES cells and MEF is needed to resolve this issue.
Various methods are available to characterize different aspects of exosomes, including their molecular cargo and their shape and size. First, exosomes are known to carry various forms of genetic material, including mRNA, miRNA, and DNA (Jabalee, Towle, & Garnis, 2018; Lázaro-Ibáñez et al., 2014; Valadi et al., 2007). To characterize experimental exosomes, RT-PCR could be used to quantify additional miRNAs, including the other members of the miR-200 family, as well as mRNA and possibly even exosomal DNA. RT-PCR gives a necessarily narrow window on the exosomal “genome” because it requires selection of primers for individual nucleic acids of interest. However, it can still be a useful tool to explore nucleic acids related to specific phenomena like apoptosis or EMT. Total RNA sequencing would represent a more holistic approach to exploring exosomal genetic material, although the technique also requires sophisticated bioinformatics techniques to glean useful data.

In addition to exploring differences in genetic material among exosome populations, it would also be useful to further explore exosomal proteins, especially those specific to the functions of the exosomes themselves. Tetraspanins, a class of transmembrane protein, are commonly used as markers for detection of exosomes, especially the tetraspanins CD9, CD63, and CD81 (Lai et al., 2010). Although specific tetraspanin populations do seem to uniquely characterize exosomes, other proteins may be more important to docking and cargo transfer to recipient cells. In particular, ECM proteins such as laminin, fibronectin, ICAM-1, and VCAM-1, and various proteoglycans seem to be important to determine to which specific cell types exosomes can dock as well as to which tissues exosomes will localize in vivo (French et al., 2017). Characterization of these proteins may give insight into specific similarities between the ES and MEF exosomes, as well as details into their interactions with cancer cells. Such
characterization could be accomplished through Western blotting of individual proteins or proteomics of the entire exosome “proteome.”

While genetic material or functional proteins can be used to differentiate between exosomes, it is equally important to be able to distinguish exosomes from other extracellular vesicles (EVs). Cells release many types of EVs that differ in size and composition. Especially in conjunction with molecular analysis, measurements of the size and shape of experimental EVs are important to confirming the identity of these particles as exosomes. To measure the size of exosomes and other small vesicles, nanoparticle tracking analysis (NTA) is an effective and simple technique. NTA uses laser light scattering to track particles’ Brownian motion, allowing one to accurately count and size EVs down to a diameter of ~50 nm (Carnell-Morris, Tannetta, Siupa, Hole, & Dragovic, 2017). Matching the measured sizes of EVs in solution to reported data would help confirm the validity of any results of experiments involving exosomes. Likewise, electron microscopy can be used to visualize exosome size as well as shape. Although exosomes had been thought to be cup-shaped or concave, analogous to a deflated basketball, newer evidence suggests that any concavity was due to distortion and that exosomes are in fact near-perfect spheres (Chernyshev et al., 2015). If EVs used in experimentation are indeed exosomes, electron microscopy should validate their shape.
5. Conclusion

In summary, we have demonstrated that ES exosomes enhance the cytotoxic and growth-suppressing effect of DOX in breast cancer cells. In the future, this finding may potentially translate to enhancement of DOX’s anti-tumor properties. While several issues related to the mechanism of cell killing need to be resolved—in particular the role of ES exosomal miR-200-family miRNAs—our results appear promising for the potential used of ES exosomes to attenuate not only DOX-induced cardiotoxicity but also muscle atrophy and dysfunction associated with chemotherapy. Moreover, studies on the beneficial effects of stem cell-derived exosomes in health and disease is a growing area of investigation which has the significant advantage of being inherently cell-free. Therefore, stem cell-derived exosomes may be attractive for future therapeutic options in cancer chemotherapy.
6. References


Reviews Cancer, 3(6), 401–410. https://doi.org/10.1038/nrc1093


Bishayee, S. (2000). Role of conformational alteration in the epidermal growth factor receptor (EGFR) function. Biochemical Pharmacology, 60(8), 1217–1223. https://doi.org/10.1016/S0006-2952(00)00425-1


ES EXOSOMES INCREASE DOX ACTIVITY IN BREAST CANCER

Analytical and Bioanalytical Chemistry, 407(12), 3285–3301.
https://doi.org/10.1007/s00216-015-8535-3

https://doi.org/10.1016/S0968-0004(98)01236-5


https://doi.org/10.1126/science.7521539


https://doi.org/10.1186/s12943-019-1029-8


https://doi.org/10.1038/ncb1722

https://doi.org/10.1007/s12079-019-00524-8

https://doi.org/10.1007/s00018-019-03020-9


ES EXOSOMES INCREASE DOX ACTIVITY IN BREAST CANCER

8674(00)80108-7


Maas, S. L. N., Breakefield, X. O., & Weaver, A. M. (2017, March 1). Extracellular Vesicles:


https://doi.org/10.1002/dvdy.24596


Retrieved from http://cancerres.aacrjournals.org/content/56/11/2527.short


ES EXOSOMES INCREASE DOX ACTIVITY IN BREAST CANCER

https://doi.org/10.1056/NEJM198010093031511


https://doi.org/10.1002/0471142735.ima03bs21


https://doi.org/10.1371/journal.pcbi.0030161


https://doi.org/10.1016/J.CELL.2009.11.007


Current Challenges in Cancer Treatment. *Clinical Therapeutics, 38*(7), 1551–1566.

https://doi.org/10.1016/j.clinthera.2016.03.026

7. Appendix

7.1. Cell Death Assay Data

**Figure 1a.** Cell death in MCF7 cancer cells, measured via trypan blue dye exclusion assay 48 hours following treatment.

**Figure 1b.** Cell death in MDA-MB-231 cancer cells, measured via trypan blue dye exclusion assay 48 hours following treatment.

Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
7.2. Flow Cytometry Data

Figure 2. Apoptosis and necrosis of MCF7 cells 48 hours following treatment. Percentages indicate percent of total cells counted in each quadrant. Q1: Necrotic cells. Q2: Late-stage apoptotic cells. Q3: Viable cells. Q4: Early-stage apoptotic cells. X-axis represents annexin V intensity; y-axis represents propidium iodide intensity. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
Figure 3. Apoptosis and necrosis of MDA-MB-231 cells 48 hours following treatment. Percentages indicate percent of total cells counted in each quadrant. Q1: Necrotic cells. Q2: Late-stage apoptotic cells. Q3: Viable cells. Q4: Early-stage apoptotic cells. X-axis represents annexin V intensity; y-axis represents propidium iodide intensity. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
**Figure 4.** Apoptosis and necrosis of MDA-MB-468 cells 48 hours following treatment. Percentages indicate percent of total cells counted in each quadrant. Q1: Necrotic cells. Q2: Late-stage apoptotic cells. Q3: Viable cells. Q4: Early-stage apoptotic cells. X-axis represents annexin V intensity; y-axis represents propidium iodide intensity. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
Table 1. Flow cytometry distributions. “Viable” refers to cells in quadrant 3, “early apoptosis” to cells in quadrant 4, “late apoptosis” to cells in quadrant 2, and “necrosis” to cells in quadrant 1.

Figure 5a. MCF7 cell line. Graphical representation of the flow cytometry distributions presented in Table 1.
**Figure 5b.** MDA-MB-231 cell line. Graphical representation of the flow cytometry distributions presented in Table 1.

**Figure 5c.** MDA-MB-468 cell line. Graphical representation of the flow cytometry distributions presented in Table 1.
7.3. Cell Proliferation Assay Data

(a)

**Figure 6a.** MTS assay of MCF7 cells 48 hours following treatment. Concentrations listed with ES, MEF, DOX+ES, and DOX+MEF conditions refer to concentration of exosomes. All treatments involving DOX used a concentration of 1 μM. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.

(b)

**Figure 6b.** MTS assay of MDA-MB-231 cells 48 hours following treatment. Concentrations listed with ES, MEF, DOX+ES, and DOX+MEF conditions refer to concentration of exosomes. All treatments involving DOX used a concentration of 1 μM. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
Figure 6c. MTS assay of MDA-MB-468 cells 48 hours following treatment. N=14 for control, DOX conditions; n=16 for ES, MEF, DOX+ES, and DOX+MEF conditions.

Note: Measurements are normalized to mean control values. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts
7.4. Real Time PCR Data

(a) miR-200c MCF7

(b) miR-200c MDA-MB-231

(c) miR-200c MDA-MB-468

Figure 7a. Real time PCR measurements of miR-200c expression by MCF7 cells 48 hours following treatment with DOX, ES, MEF, DOX+ES, and DOX+MEF.

Figure 7b. Real time PCR measurements of miR-200c expression by MDA-MB-231 cells 48 hours following treatment.

Figure 7c. Real time PCR measurements of miR-200c expression by MDA-MB-468 cells 48 hours following treatment.

Note: Columns represent fold change compared to control using the $2^{-\Delta\Delta CT}$ method and normalized to snoRNA-202.

Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
7.5. Western Blot Data

Figure 8a. Expression of Bax by MCF7 cells 48 hours following treatment with DOX, ES, DOX+ES, and DOX+MEF (n=2).

Figure 8b. Expression of Bcl-2 by MCF7 cells 48 hours following treatment (n=2).

Figure 8c. Ratio of Bax to Bcl-2 expression by MCF7 cells (n=2).

Figure 8d. Representative Western blot exposures showing expression of Bax and Bcl-2 in MCF7 cells.

Note: GAPDH was used as a house-keeping protein for loading control. Values are relative to the mean of control values, normalized to GAPDH. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
Figure 9a. Expression of Bax by MDA-MB-231 cells 48 hours following treatment with DOX, ES, MEF, DOX+ES, and DOX+MEF (n=2).

Figure 9b. Expression of Bcl-2 by MDA-MB-231 cells 48 hours following treatment (n=2).

Figure 9c. Ratio of Bax to Bcl-2 expression by MDA-MB-231 cells (n=2).

Figure 9d. Representative Western blot exposures showing expression of Bax and Bcl-2 in MCF7 cells.

Note: GAPDH was used as a house-keeping protein for loading control. Values are relative to the mean of control values, normalized to GAPDH. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
Figure 10a. Expression of Bax by MDA-MB-468 cells 48 hours following treatment with DOX, ES, DOX+ES, and DOX+MEF (n=2).

Figure 10b. Expression of Bcl-2 by MDA-MB-468 cells 48 hours following treatment (n=2).

Figure 10c. Ratio of Bax to Bcl-2 expression by MDA-MB-468 cells (n=2).

Figure 10d. Representative Western blot exposures showing expression of Bax and Bcl-2 in MCF7 cells.

Note: GAPDH was used as a house-keeping protein for loading control. Values are relative to the mean of control values, normalized to GAPDH. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
7.6. Cell Micrographs (Transmission Light Microscopy)

<table>
<thead>
<tr>
<th>Control</th>
<th>ES</th>
<th>MEF</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Control" /></td>
<td><img src="image" alt="ES" /></td>
<td><img src="image" alt="MEF" /></td>
</tr>
<tr>
<td><img src="image" alt="DOX" /></td>
<td><img src="image" alt="DOX+ES" /></td>
<td><img src="image" alt="DOX+MEF" /></td>
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**Figure 11.** Representative transmission light micrographs of MCF7 cells plated in wells of a 96-well plate. Micrographs were taken 48 hours following treatment with DOX, ES, MEF, DOX+ES and DOX+MEF. Note that the cells are firmly attached in the Control, ES and MEF groups with fewer floating (dead) cells. Treatments with DOX show a large number of floating rounded dead cells which were significantly less in the DOX+ES group compared to DOX alone or DOX+MEF groups. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
Figure 12. Transmission light micrographs of MDA-MB-231 cells plated in wells of a 96-well plate. Micrographs were taken 48 hours following treatment with DOX, ES, MEF, DOX+ES, and DOX+MEF. Note that the cells are firmly attached in the Control, ES and MEF groups with fewer floating (dead) cells. Treatments with DOX show a large number of floating, rounded dead cells (and fewer viable cells) which were significantly less in the DOX+ES group compared to DOX alone or DOX+MEF groups. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblasts.
Figure 13. Transmission light micrographs of MDA-MB-468 cells plated in wells of a 96-well plate. Micrographs were taken 48 hours following treatment with DOX, ES, MEF, DOX+ES, and DOX+MEF. Note that the cells are firmly attached in the Control, ES and MEF groups with fewer floating (dead) cells. Treatments with DOX show a large number of floating, rounded dead cells (and fewer viable cells) which were significantly less in the DOX+ES group compared to DOX alone or DOX+MEF groups. Abbreviations: DOX: Doxorubicin; ES: Embryonic stem cell; MEF: Mouse embryonic fibroblast.