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Characterization of Exosomes and Their Role in Cellular Migration in Chemosensitive and Chemoresistant Ovarian Cancer Cells

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1. Abstract

Chemotherapy-resistant cells that remain after primary cancer treatment lead to recurrence and metastasis, resulting in a poor prognosis for patients. Chemoresistant cells were created by our collaborators using the human ovarian cancer cell line, OVCAR8, by selection of cells that survived serial culture with cisplatin, a platinum-based chemotherapeutic. The cisplatin-resistant cells (OVCAR8R) demonstrate a morphology unique from the original cisplatin-sensitive cells (OVCAR8) which could correlate with their difference in aggressiveness. My research goal is to isolate and characterize exosomes from the OVCAR8 and OVCAR8R lines. Exosomes are small vesicles secreted from cells and contribute to the tumor microenvironment (TME). It has been found that exosomes secreted from cancerous cells hold the ability to influence neighboring cells and increase their metastatic ability. Exosomes contain a unique signature of packaged proteins and RNA that may be responsible for their role in cancer progression. The goal of these experiments is to determine the differences between the exosomal cargo derived from chemosensitive and chemoresistant ovarian cancer cells as well as the effects that exosomes isolated from each line have on promoting cellular migration. Exosome isolation and characterization could be employed as a noninvasive diagnostic tool to identify early-stage ovarian cancer.

2. Introduction

2.1 Exosomes and Ovarian Cancer

Over the past five years, exosomes have become an attractive target in cancer research. Exosomes are small single-membrane extracellular vesicles between 30 and 150 nm in size which are secreted from all cells in the body [1]. Although the mechanism and exact biological function of exosomes is unclear, it has been found that exosomes that are secreted into the tumor microenvironment (TME) are taken up by neighboring cells and influence their behavior [2]. Exosomes contain proteins and RNA that play a role in increasing aggressiveness in cancer cells and may influence an increased aggression in neighboring cells. Exosomes are able to set the TME up for metastasis by enabling the escape of tumor cells by stopping the immune systems surveillance and are thereby set up the secondary site for tumor cells to attach [7]. Because of this ability, researchers have talked about the possibility of exosomes as a system for drug delivery [7]. Although the current study is focused on ovarian cancer, there are numerous other cancers that have been studied surrounding the influence of exosomes in their progression. Exosomes have been linked to tumorigenesis through chemoresistance, angiogenesis, and epithelial-mesenchymal transition (EMT). Therefore, the possibility of targeting exosomes as a form of cancer treatment is promising, but more data to determine the specific role of exosomes in cancer is needed. Exosome isolation and characterization could be employed as a noninvasive diagnostic tool to identify early-stage ovarian cancer.

Exosomes play a role in both metastasis and drug resistance. A tumor microenvironment suitable for the specific type of cancer is needed for cancer cells to proliferate and survive. Exosomes are able to create this type of environment through numerous pathways that promote angiogenesis, metastasis, and EMT [10]. Exosomes are released more in stressful conditions in order to remodel the TME and make it a suitable environment [10]. Studies found that eliminating

exosomes from the TME decreased cancer progression [10]. Exosomes contain proteins that are common to all cell types and proteins that are specific to certain cell and tissue types [10]. The proteins from specific types of cells increase the metastasis of cancers to specific secondary sites. Exosomes also contain specific proteins involved in angiogenesis such as vascular endothelial growth factor, or VEGF, and DLL4 which plays a role in the Notch pathway that promotes angiogenesis [10]. There are also several proteins involved in EMT that have been found to be in exosomes including TGF-β, β-catenin, and Caveolin-1 [10]. With regard to drug resistance, drug resistant cancers can transport anti-cancer drugs out of the cell through exosomes which can then transition this resistance to drug sensitive cells in the TME [10].

Ovarian cancer is a common gynecological cancer and the deadliest cancer to women in the US because of its aggressiveness and its metastatic potential [1]. 75% of ovarian cancers are caught in late stages due to their location and ability to form a large tumor in the peritoneal cavity before diagnosis. Surgery and platinum-based chemotherapy are the main treatments for ovarian cancer, but resistance to cisplatin creates a major clinical problem whether it occurs before or after treatment [7]. This acquired resistance resulting in relapse occurs in about 50% of patients within five years of diagnosis [13]. Cells that become resistant also acquire advanced mechanisms that allow them to become more aggressive by increasing their metastatic ability leading to a poor prognosis [8]. Some of the secondary sites that ovarian cancer will spread after acquiring resistance are the bowel, bladder, and liver [13].

Circulating miRNAs within exosomes have been found to play a role in this establishment of chemoresistance in ovarian cancer. Previous studies have also identified models that can be used to monitor cancer progression and effectiveness of treatment based on the exosomes secreted from these cancer cells [13]. Exosomes secreted from ovarian cancer cells influence neighboring cells through signaling in the tumor microenvironment (TME) and eventually may circulate around the

body to promote metastasis to secondary sites by creating a suitable environment [13]. Exosomes are also able to evade chemotherapeutic effects because of their ability to regulate reactive oxygen species concentrations which could be a part of their mechanism in promoting aggressive cancer [13]. A previous study analyzed a liquid biopsy of ovarian cancer and determined the exosomal components including miRNA. This study supports liquid biopsies and circulating exosomes as a possible diagnostic tool for ovarian cancer which may promote better patient survival and improve the prognosis for patients [19].

Exosomes as vesicles also play a role in chemoresistance in ovarian cancer cells. Previous studies have found that exosomes are able to package chemotherapeutic agents and flush them out of cancer cells [13]. Additionally, exosomes promote chemoresistance when secreted from a chemoresistant cell to influence neighboring cells by changing their phenotypes and protein expression that leads to more aggressive cancer [13]. Finally, exosomes are thought to be able to promote angiogenesis when secreted from chemoresistant ovarian cancer cells by overexpressing miR-130a [18]. The main clinical goal of studying exosomes is focusing on providing patients with an earlier diagnosis using predictive biomarkers, including those contained in exosomes, in order to improve survival rates.

2.2 Exosomes in Various Cancers

Recently, a greater understanding of exosomes and their role in different pathways and methods of action have been determined. In colorectal cancer, chemoresistance becomes a problem after treatment. One cell type in the TME that promotes cancer through communication with surrounding cells and also controls the extracellular matrix (ECM) are cancer associated fibroblasts (CAFs) [3]. The exosomes secreted from CAFs have been found to promote metastasis by setting up the secondary site as a suitable environment for these cells to inhabit and increase resistance in colorectal cancer cells. Using Western blots, transwell assays, and migration assays, researchers

found that exosomes secreted from CAFs are able to transfer miRNAs between cells which play a role in EMT, metastasis, and apoptosis [3]. These miRNAs are able to inhibit proteins that increase the aggressiveness of colorectal cancer. Two of these proteins are FBXW7 which is a tumor suppressor gene and MOAP1, or modulator of apoptosis 1, which associates with Bax to promote apoptosis [3]. Understanding the numerous proteins contained in these exosomes in the TME that influence surrounding cells to promote metastasis is important in finding possible treatments for these cancers [3]. Another study examined a specific miRNA called miR-205, which is found in exosomes of ovarian cancer cells, and promotes metastasis through angiogenesis, the ability of tumor cells to recruit oxygen and nutrients for survival as the tumor grows [2]. After analysis of multiple proteins and miRNAs, it was found that miR-205 was upregulated in metastatic cancers and released in exosomes where it was found to easily be transported to surrounding cells and increase their aggressiveness, especially in surrounding epithelial cells [2]. This miRNA was also analyzed in a mouse model determining that miR-205 played a role in angiogenesis through the PTEN-AKT pathway which could be a promising pathway to target for possible treatment [2].

Another important pathway shown to involve exosomes and cancer progression is the TGF-β/Smad signaling pathway. A study focusing on this pathway found that exosomes use this pathway in order to regulate the TME [4]. Four liver cell lines were used to determine exosome contents through Western blot as well as to study their role in migration through wound healing and transwell assays. After culturing recipient cells with exosomes isolated from cells with either low metastatic potential or high metastatic potential cancer, it was determined that cells cultured with the more aggressive exosomes were more capable of migration and invasion [4]. Expression of E-cadherin, vimentin, TGF-β, and Smad were also analyzed in these exosomes to determine if these proteins played a role in aggressiveness. E-cadherin is a cell adhesion molecule and decreased levels show increased EMT and movement of cells from epithelial tissue to mesenchymal which makes them more metastatic. Vimentin is a structural protein and is expressed in mesenchymal

cells and drives EMT in cancer cells. TGF-β is a cytokine that signals the Smad complex to form which then continues down the pathway and allows for increased transcription in the nucleus. It was found that the highly metastatic cancer derived exosomes had increased TGF-β and that this pathway allows exosomes to increase EMT in surrounding cells [4]. A similar study also assessed how culturing exosomes from highly metastatic ovarian cancer cells with less metastatic cancer cells increased their aggressiveness which shows that exosomes have this ability, but specific pathways or proteins that play an important role may prove to be cell-type specific [1].

A study using leukemia cell lines investigated the mechanism by which bone marrow mesenchymal stem cell (BM-MSC) exosomes increased proliferation, invasion, and chemoresistance using the protein S100A4 [5]. Past studies have shown that exosomes from more aggressive cancer cells show an upregulation of important proteins that play a role in proliferation, metastasis, and chemoresistance. This study specifically addressed one protein, S100A4, and its role in chemoresistance in acute myeloid leukemia. S100A4 is part of the S100 calcium binding protein family and has been found to increase tumorigenesis through angiogenesis [5]. Exosomes isolated from the BM-MSC cells were found to upregulate common exosomal markers like CD44, CD73, CD90, and CD105 [5]. When culturing these exosomes with leukemia cells, it was found that they promoted both migration and invasion when compared to the control [5]. Researchers then knocked down S100A4 expression and determined that when this was done, migration and invasion of the cells decreased showing that S100A4 plays a role in cancer metastasis [5]. Also, it was found exosomes from cells that express S100A4 increased chemoresistance of the leukemia cells and promoted the release of more cancerous cells into the peripheral blood for metastasis [5].

2.3 Heat Shock Proteins

Heat shock proteins (HSPs) are a family of proteins that are upregulated in cells under stressful conditions. They play a role in cellular homeostasis and are typically upregulated in cancer cells

resulting in increased cell proliferation and aggression [11]. Heat shock proteins inhibit apoptosis at several steps in the apoptotic cascade. The small heat shock protein αB-crystallin inhibits both the mitochondrial- and receptor-dependent apoptotic pathways by disrupting the activation of caspase-3, while HSP27 binds to cytochrome c, disrupting the mitochondrial-dependent apoptotic pathway [17]. HSP27 is involved with both inhibiting apoptosis as well as preventing the aggregation of misfolded proteins [11]. Therefore, overexpression of αB-crystallin and HSP27 may underlie the resistance of cancer cells to cytotoxic agents as it enables the cell to avert apoptosis through caspase inactivation. HSPs are involved in inhibiting apoptosis, angiogenesis, metastasis, and resistance. When found in exosomes, HSPs can be used as cancer biomarkers [11]. HSP70 and HSP27 are two of the most common heat shock proteins found in exosomes. HSP70 is an anti-apoptotic protein by blocking pathways involved in apoptosis [11]. HSP70 is a large heat shock protein located on the outer membrane of exosomes which could support its role in cell-to-cell communication to increase aggressiveness in surrounding cells [20]. HSP70 and HSP27 are located in both the membrane and lumen of exosomes [11]. It was also found in previous studies that an increased expression of heat shock proteins in exosomes correlated with the metastatic potential of the cancer [11]. The role of heat shock proteins and their expression in exosomes as well as their role in chemoresistance is not well known [20].

Previous lab members analyzed our chemosensitive (OVCAR8) and chemoresistant (OVCAR8R) cell lines for heat shock proteins. Both αB-Crystallin and HSP27 were found to be constitutively expressed in the cisplatin-resistant ovarian cancer cell line (OVCAR8R) as compared to its cisplatin-sensitive counterpart (OVCAR8). This supports that overexpression of heat shock proteins can confer resistance to chemotherapy and promote a more aggressive tumor type. The role of heat shock proteins has not been extensively studied in ovarian cancer and the release of small heat shock proteins in exosomes in cancer cells is unknown. Of interest, αB-crystallin has been shown to localize within mitochondria and exosomes in retinal pigment epithelial (RPE) cells

[14, 15], and released by astrocytes [16], however, few studies have addressed the secretion of αB-crystallin from cancer cells. Since the majority of ovarian tumors eventually recur in a drug-resistant form leaving patients few treatment options, the goal of this study was to determine the differences between the exosomal cargo derived from chemosensitive and chemoresistant ovarian cancer cells as well as the effect of exosomes on cellular migration.

3. Materials & Methods

3.1 Cell Culture Maintenance

The ovarian cancer cell lines, OVCAR8 (Cisplatin Sensitive) and OVCAR8R (Cisplatin Resistant) were provided by our collaborator (Shanta Messerli) and were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin streptomycin. Cells were maintained and incubated at 37°C in a 5% CO_2 environment. OVCAR8R cells were maintained in 5mM Carboplatin (Sigma).

3.2 Exosome Isolation

Exosomes can be isolated from cell culture supernatant using an exosome isolation kit or by ultra-centrifugation [6]. Exosomes were isolated using the Total Exosome Isolation protocol (Invitrogen). Cell culture media from ovarian cancer cell lines, OVCAR8 (Cisplatin Sensitive) and OVCAR8R (Cisplatin Resistant), was collected and centrifuged at 2000 x g for 30 minutes. The supernatant was then moved to a new tube and Total Exosome Isolation reagent was added in a 1:2 ratio. Samples were incubated overnight at 4°C. Samples were then centrifuged at 10, 000 x g for one hour and supernatant was discarded. Exosome pellet was then resuspended in 75 µL 1X PBS and 75 µL Laemmli buffer and stored in -20°C for further characterization.

3.3 Cell Harvest and Western Blot Analysis

Cells were treated as described, then SDS-PAGE was performed on whole-cell lysates and proteins were transferred to a PVDF membrane. The membrane was blocked in 10 mL (5% milk in Tris-Buffered Saline Tween-20, TBST) for 1 hour. The membrane was washed 4 times in TBST for 5 minutes and blocked in 5% Milk-TBST. Membranes were incubated for 1 hour each in primary antibodies diluted in 1% milk in TBST. Primary antibodies included the following: 1:1000 dilution of Hsp27 (SPA-800), Hsp70 (SPA-822), or αB-Crystallin (SPA-222) (Enzo), Flotillin (D2V7J) (Cell Signaling); 1:2500 dilution of anti-Tubulin (Sigma, T5168), 1:500 dilution of VEGF (C-1) (Santa Cruz Biotechnology), or Vimentin (D21H3) (Cell Signaling). primary antibody dilutions. The membrane was washed 4 times in TBST for 5 minutes and incubated at room temperature for 1 hour on a rocker in 10 mL (1% milk in TBST) 1:10,000 secondary HRP-conjugated anti-rabbit antibody dilution (SC-2004 Santa Cruz Biotechnology) or 1:10,000 secondary HRP-conjugated anti-mouse antibody dilution (SC-2005 Santa Cruz Biotechnology). The membrane was washed 4 times with TBST for 5 minutes, then once in dH_2O for 5 minutes, followed by incubation in 10 mL ECL Chemiluminescence for 2 minutes and exposed to film.

3.4 EMT and Transwell Migration Assay

Epithelial-mesenchymal transition (EMT) is a hallmark of cancer that refers to the development of cancer cells from an epithelial to mesenchymal phenotype, which increases their migratory ability and allows for the cancer to metastasize [9]. During this transition, cancer cells gain characteristics that allow for increased motility and decreased adhesion which allows them to lift off the extracellular matrix, enter a blood vessel, and travel to a distant location that is suitable for these cells. They then exit the blood vessel and settle down [9]. Each cancer cell type metastasizes to specific organs because of this need for a suitable tumor environment. In recent years, exosomes have become a possible answer to the question of how these cancer cells gain mesenchymal characteristics in order to move to a secondary site. Both miRNAs and TGF-β in exosomes have

been linked to cancer progression and may play a role in EMT of cancer cells [4, 9]. In the research laboratory, transwell migration assays are used to measure the migratory ability of cells. Cells are plated in the top chamber of a well and are allowed to migrate through a thin membrane towards a chemoattractant, which most typically is medium with increased growth factors as compared to the top chamber Transwell migration assays are a new method in our lab so as these experiments were done, a protocol was designed for future students (see 6. Appendix).

The ovarian cancer cell lines, OVCAR8 (Cisplatin Sensitive) and OVCAR8R (Cisplatin Resistant) cells were plated at 75,000 cells per well in the top chamber of a six well plate with a transwell insert (8.0 mm, Corning) that had been equilibrated for two hours in 10% FBS-containing DMEM. 2.5 mL of DMEM with 20% FBS was added to the bottom chamber to promote migration. 150 µL of either the control (PBS free) or exosomes were added to respective wells. Cells were incubated in the 37°C incubator for either 24 hours or 48 hours. The top chambers were swabbed to remove nonmigrating cells. The cells that had migrated through to the lower membrane were then washed twice with PBS, fixed with 100% methanol, and stained with crystal violet. Deionized water washes were done until all of the crystal violet was washed out. Eight images per well were then taken using an inverted microscope. Cells from each of the eight fields were counted to determine the migration differences between each cell line and whether exosomes impacted migration. (See appendix).

4. Results and Discussion

4.1 Morphological and Protein Analysis of OVCAR8 and OVCAR8R cell lines

Previous work in our lab characterized two cell lines that were created by Dr. Shanta Messerli and colleagues. The morphological and molecular traits involved in the resistance of cells to cisplatin, untreated OVCAR8, and OVCAR8R cells were analyzed for morphology and expression of HSPs, Caspase-3, and Vimentin. Cisplatin-resistant OVCAR8R cells appear elongated and spread out on the

surface of the flask, whereas the Cisplatin-sensitive OVCAR8 cells appear smaller and are more spindle-shaped (Carmichael, Honors Thesis, 2020, Figure 1A). Expression of HSP27 and αB-Crystallin was not detected in the OVCAR8 cell line, however, both HSP27 and αB-Crystallin are constitutively expressed in OVCAR8R (Figure 1B). HSP overexpression correlates with expression of vimentin, a well-characterized marker that supports a more metastatic phenotype of the chemoresistant cells (OVCAR8R) (Figure 1B). Furthermore, the resistant cells express lower levels of caspase-3 which supports they are less likely to enter into an apoptotic program as compared to their cisplatin-sensitive counterpart (Figure 1B).

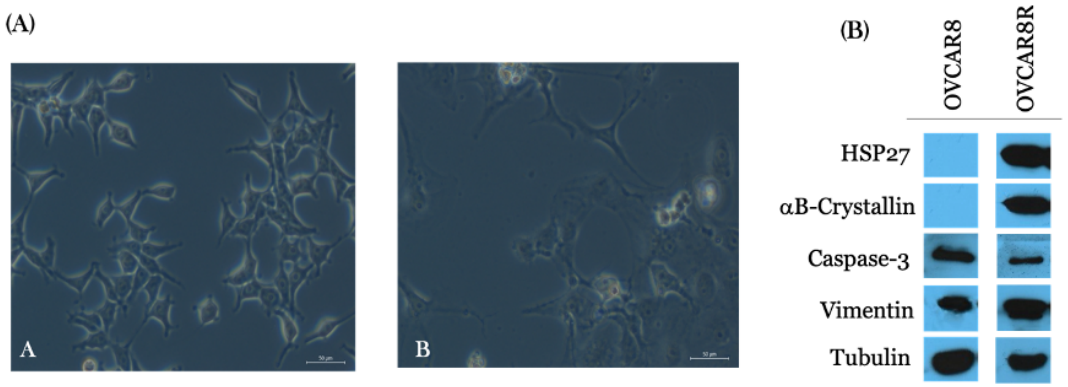


Figure 1: (a) Morphological differences of OVCAR8 and OVCAR8R cells respectively. (b) Western blot showing protein differences between the OVCAR8 and OVCAR8R cells including HSP27, αB-Crystallin, Vimentin, and Capspase-3.

In order to determine if treatment with a chemotherapeutic agent would alter exosomal secretion and protein expression, OVCAR8 and OVCAR8R cell lines were treated with 5 μm Cisplatin or 2.5 μm Withaferin A (WA). Total cell lysates were prepared along with isolation of exosomes which were subject to SDS PAGE and Western blot analysis to characterize protein expression. LAMP, or lysosome associated membrane glycoprotein, maintains the membrane integrity of lysosomes, and was upregulated in the OVCAR8R lysates (Figure 2). An upregulation of LAMP in the OVCAR8R cell

line may correlate with chemoresistance [21]. LAMP1 has been implicated in cancer development and progression. Indeed, LAMP1 expression in breast cancer is associated with malignant attributes of this cancer type and as such, our results support that LAMP1 expression is an important prognostic factor for breast and potentially other cancer subtypes such as chemoresistant ovarian cancer [21]. VEGF, or vascular endothelial growth factor, mediates angiogenesis, the formation of blood vessels. Here, an upregulation of VEGF in chemoresistant cells supports the idea that their angiogenic ability increases their aggressiveness (Figure 2). Furthermore, the expression of VEGF was decreased in both cell lines that were treated with chemotherapeutic agents indicating that these agents may decrease markers that promote angiogenic potential. This finding was supported by previous work in which an ELISA from conditioned medium from OVCAR8 and OVCAR8R cells showed increased VEGF secretion in OVCAR8R as compared to OVCAR8. This is a preliminary result that supports increased expression of VEGF in OVCAR8R cell lysates, however, this work needs to be repeated (data not shown).

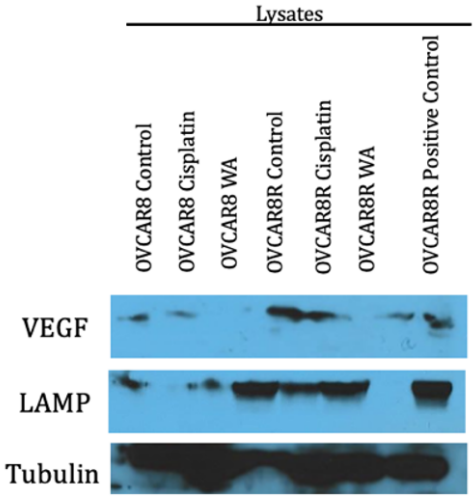


Figure 2: Protein expression from total cell lysates from cell incubated with vehicle (DMSO), Cisplatin, or Withaferin A (WA) was determined via Western blot analysis. Expression of LAMP and VEGF was increased in OVCAR8R as compared to OVCAR8. VEGF expression was downregulated in both cell lines that were treated with chemotherapeutic agents.

4.2 Protein Analysis of OVCAR8 and OVCAR8R Exosomes

After isolation of exosomes from both OVCAR8 and OVCAR8R lines, the exosomes were analyzed using Western blot analysis for various proteins associated with cancer progression. Initial experiments to characterize exosomes analyzed Alix, a common exosomal marker, expression in exosomes but was inconclusive so we chose to explore another common marker, Flotillin-1. In order to ensure that the exosomes analyzed were only from the OVCAR8 and OVCAR8R cells, cells were switched from DMEM with normal Fetal Bovine Serum (FBS) to an exosome depleted FBS (purchased with an ATP grant) for all subsequent experiments.

HSP70 and Flotillin-1 were shown to be expressed in all exosomes (Figure 3). Flotillin-1 was expected because of its role in forming caveolae on the inner cell membrane that is used for signal transduction. Flotillin-1 is a common exosome marker and can be a way to determine if the isolation of exosomes was completed before further analysis. HSP70 is upregulated in cancer cells when chemoresistance is acquired and has been found to be secreted from exosomes as a danger signal because of their ability to increase metastasis and evade the immune response. This supports the idea that detecting HSP70 in exosomes could be a diagnostic tool. LAMP, HSP27, and αB-crystallin were not expressed in exosomes but were expressed constitutively in the OVCAR8R control lysate (Figure 3). LAMP is involved in maintaining lysosomal integrity so was not expected to be secreted in exosomes. HSP27 inhibits apoptosis and is expressed in the chemoresistant cell line. αB-crystallin functions as a chaperone that refolds misfolded proteins and inhibits apoptosis and has been shown to be abundantly expressed in the chemoresistant cell line. Despite that other reports support that small HSP's may be secreted to promote an immune response, this experiment shows that exosomes lack expression of both HSP27 and αB-crystallin. This is a novel finding to show that these specific markers of a more chemoresistant phenotype are not exported into cellular exosomes. Previous studies showed HSP27 and αB-crystallin in exosomes from other cell types, so

running this experiment again to confirm this finding would be beneficial. Future experiments will analyze CD63 and TGF-β as these proteins were shown to be packaged into exosomes in more chemoresistant and aggressive cell types.

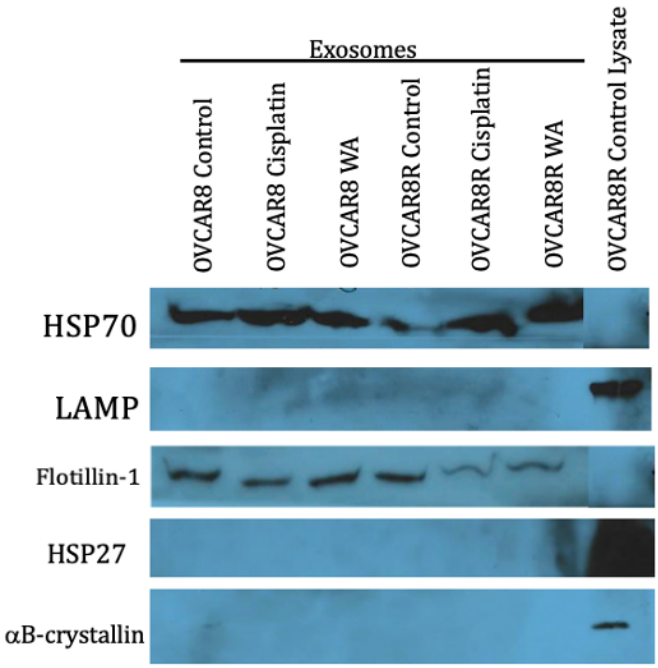


Figure 3: HSP70 and Flotillin-1 were expressed in both OVCAR8 and OVCAR8R exosomes but not the OVCAR8R control lysate. LAMP, HSP27, and αB-crystallin were not expressed in exosomes but were expressed in the OVCAR8R control lysate.

4.3 Migration of OVCAR8 and OVCAR8R Cell Lines

To determine the differences in migratory ability, two transwell migration assays were done. The OVCAR8 and OVCAR8R cells were plated with either PBS (control) or exosomes isolated from, OVCAR8 or OVCAR8R as previously discussed. Briefly, in order to isolate exosomes from exponentially growing cells, OVCAR8 and OVCAR8R cells were plated in T25 flasks and allowed to grow for 48 or 72 hours. Cells were counted and exosomes were either collected into PBS for

delivery to recipient cells or into 2xSDS-PAGE Buffer in order to ensure that isolated exosomes were normalized for an equal number of cells per final volume for each assay. Cells were then plated with exosomes plating in the top chamber of a transwell chamber and migration was analyzed after 24h or 48h. A pilot experiment showed an increased number of migrated cells in the OVCAR8R cell line compared to the OVCAR8 cell line (Figure 4). The OVCAR8 cells cultured with the OVCAR8R exosomes had an increased number of migrated cells compared to both the control and the OVCAR8 cells with the OVCAR8 exosomes (Figure 4).

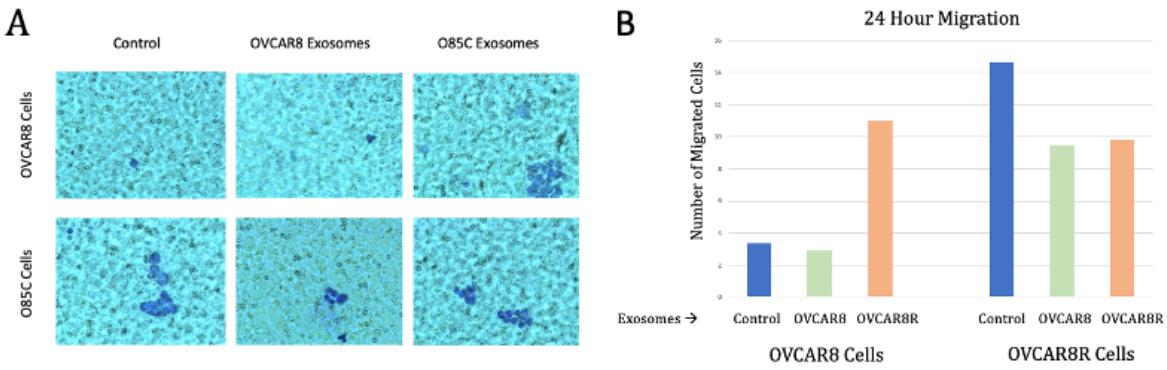


Figure 4: Number of cells that migrated through a transwell membrane was measured after; cells were plated in the top chamber and incubated with no exosomes or exosomes isolated from OVCAR8 or OVCAR8R cells.

Cells were then allowed to migrate toward 20% FBS in the bottom chamber, then fixed and stained with Crystal violet after 24h. (a) A representative image of cells migrated to the underside of a transwell membrane 24h after plating (OVCAR8, OVCAR8R, with or without exosomes). (b) Exosomes from the Cisplatin-resistant OVCAR8R cells increased migration capability of OVCAR8 cells as compared to exosomes from cisplatin-sensitive OVCAR8 cells. All OVCAR8R cells with or without exosomes migrated more readily than OVCAR8 Cells.

Because the numbers overall were low from the first transwell migration assay, a second experiment was done with an increased concentration of exosomes as well as allowing a longer

time (48 hours) for migration compared to the original 24 hours. These results also showed an increased migratory ability of OVCAR8 cells with OVCAR8R exosomes (Figure 5). It also appeared that OVCAR8R cells cultured with OVCAR8 exosomes had a decreased number of migrated cells compared to the OVCAR8R control (Figure 5). While these studies are preliminary, this supports that the exosomes from a more aggressive and chemoresistant cell line can influence the migration of a more chemosensitive cell line. Further work will allow statistical analysis of this finding.

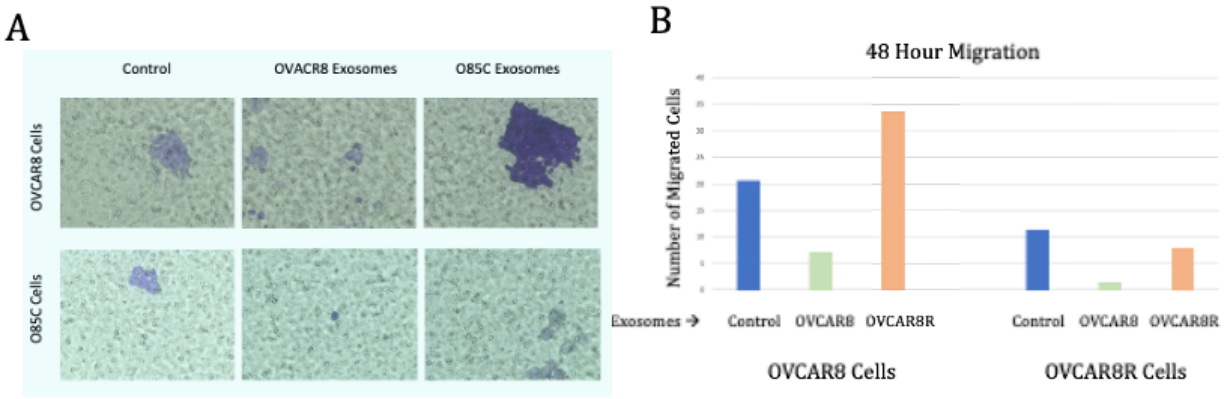


Figure 5: Number of cells that migrated through a transwell membrane was measured after; cells were plated in the top chamber and incubated with no exosomes or exosomes isolated from OVCAR8 or OVCAR8R cells.

Cells were then allowed to migrate toward 20% FBS in the bottom chamber, then fixed and stained with Crystal violet after 48 hours. (a) A representative image of cells migrated to the underside of a transwell membrane 24h after plating (OVCAR8, OVCAR8R, with or without exosomes). (b) Exosomes from the Cisplatin-resistant OVCAR8R cells increased migration capability of OVCAR8 cells as compared to exosomes from cisplatin-sensitive OVCAR8 cells. Exosomes from the cisplatin sensitive OVCAR8 cells decreased migration ability of OVCAR8R cells compared to exosomes from cisplatin resistant OVCAR8R cells.

During my first semesters of research, I worked to help characterize other chemoresistant cell lines (breast cancer cells) as well as to silence HSP27 and αB-crystallin in the OVCAR8R cells

via CRISPR-Cas9. Several clones of Hsp27-CRISPR-Cas9 or Hsp27/αB-crystallin-CRISPR-Cas9 double knockout cells were isolated and have yet to be characterized and studied in detail. Previous work confirms the successful silencing of HSP27 and αB-crystallin knockouts (Figure 6); a Western blot shows the successful silencing of these proteins in the OVCAR8R ovarian cancer cell line. Further experiments on these knockout clones will determine whether their exosomal profiles are unique and will determine whether exosomes from cells in which small heat shock proteins were silenced will impact cellular migration.

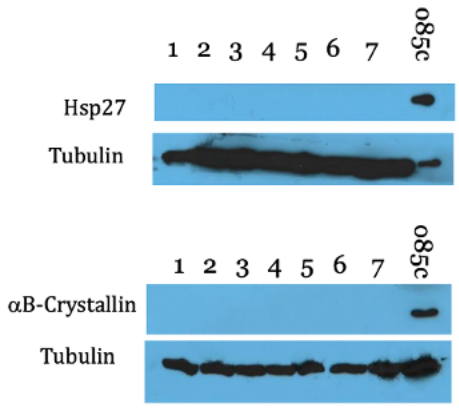


Figure 6: Western Blot Analysis after CRISPR-Cas9 targeting of Hsp27 or Hsp27-αB-Crystallin. Western blot analysis confirms seven successful Hsp27 or Hsp27-αB-Crystallin knock-out clones. A cell lysate from the OVCAR8R cells (O85C) was used as a positive control, Tubulin expression shows that equal protein is loaded in each well.

5. Conclusions and Future Work:

The protein analysis showing an upregulation of VEGF and expression of LAMP in the chemoresistant cell line is a novel finding to our lab and could be correlated with their increased aggressiveness because of the evidence of these proteins in promoting angiogenesis and aggressiveness in other cancer cell types. The protein analysis showing expression of Flotillin-1 in the exosomes which supports that the Total Exosome Isolation kit being used is in fact isolating

exosomes from these cell lines. With HSP70 being upregulated in the exosomes, this could be a possible protein to be targeted in order to help treat ovarian cancer. The results of the transwell migration assay support the hypothesis that exosomes from chemoresistant ovarian cancer cell lines are able to increase the migratory potential and aggressiveness of the chemosensitive ovarian cancer cells when cultured together. Using all of this data about the role of exosomes, what they contain, and the effect they have on migration is crucial to further steps in ovarian cancer treatments. If future experiments determine more proteins found in exosomes of ovarian cancer, this can be used as a noninvasive diagnostic tool of ovarian cancer.

Based on previous work on TGF-β and the AKT pathway [4, 2], future experiments should analyze the expression of TGF-β, Smad, and the molecules in the AKT pathway in these cell lines to determine what role they play in proliferation and if this could be a possible pathway to target in the ovarian cancer exosomes. More experiments need to be done using the transwell migration assay to gather more accurate results. Other experiments that would be interesting to run are wound healing assays and transwell invasion assays to analyze the difference between cell lines and exosome culture.

Experiments by other lab members are underway to systematically determine the specific roles of αB-crystallin and HSP27 in conferring resistance of ovarian cancer cells to apoptosis. CRISPR experiments will reveal whether silencing of αB-crystallin, HSP27, or both proteins will yield unique exosomal signatures with hopes to better understand the role of small HSPs and exosomes in migration. A study in retinal pigment epithelial cells shows that αB-crystallin is secreted in exosomes so experiments to target each small HSP will help to determine the role of each protein in a more chemoresistant and aggressive phenotype [12].

No studies have addressed the role of αB-crystallin and HSP27 as molecular players in apoptosis resistance in cisplatin resistant ovarian cancers. During my first year of research, I helped to create novel cell lines in which we used OVCAR8R cells and silenced HSP27 alone or both

HSP27 and αB-crystallin. These studies yielded several clones (seven are shown, Figure 6) for each cell line in which HSP27 and/or αB-crystallin expression was silenced following transfection with CRISPR-Cas9 constructs targeted to each gene, clone selection in puromycin and propagation of each cell line. These cell lines will allow us to clearly define the role of HSPs in chemoresistance and allow the isolation of exosomes from these cells to determine if exosomes can confer resistance to apoptosis, increased migration, metastatic potential, and angiogenesis. By understanding how the exosomes from these cell lines differ in both contents as well as migration potential, we could determine a way to target these exosomes in order to combat chemoresistance and find a treatment for ovarian cancer.

Future research will help to elucidate the role of small HSPs in exosomal trafficking as well as the effectiveness of secondary chemotherapeutics as a potential therapy for ovarian cancer cells that acquire resistance to platinum-based therapies.

6. Appendix

6.1 Transwell Migration Assay Protocol

Day 1 – 4:

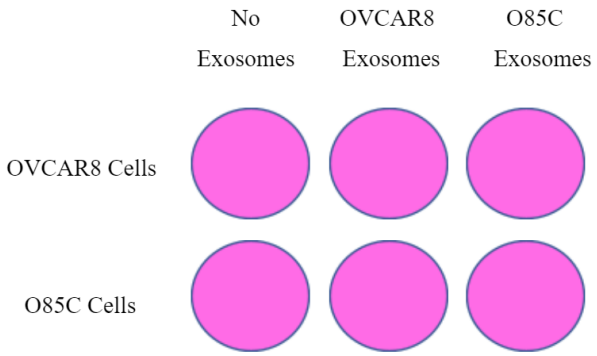
1. Set up T25 flasks with 200, 000 cells and 4 mL of exosome free DMEM. Let grow for 72 hours.
    a. Isolate exosomes from OVCAR8 and O85C lines using Total Exosome Isolation (from cell culture media) protocol
        - Count cells before adding exosome isolation buffer and compare to number of cells that were set up originally. Normalize ratio of cells to number of exosomes so at the end of the isolation there is an equal concentration of exosomes between lines.
        - When adding PBS – use PBS free, 200, 000 cells per 50 μL of PBS
        - Exosome Isolation Protocol
2. Make lysates out of OVCAR8 and O85C cell lines, keep two flasks growing of OVCAR8 and O85C to use for plate set up tomorrow.

Day 5 – 6:

1. Equilibrate transwell plate by adding 2.5 mL DMEM to bottom chamber and 1.5 mL DMEM to top. Let sit for one hour or more before use.
2. Count cells and collect 75, 000 cells
3. Centrifuge and discard media

4. Add 6mL of exosome depleted DMEM
5. Recount cells (cell number is important!) to ensure that final cell number is 75, 000 cells
6. Add 2.5 mL of 20% FBS to bottom of each well to promote migration of cells
7. Add 150 µL of exosomes to respective wells (concentration of exosomes to be 200, 000 cells per 50 µL)
    a. For control add 150 µL of PBS free
8. Add 2 mL of cells to the middle of each well. Be careful not to shake, do not want media and cells to mix.
9. Store in incubator for 48 hours

Transwell set up template:



Day 7:

1. After 48 hours, aspirate media.
    a. Wash twice with PBS (2 mL in bottom and 1.5 mL in top chamber)
1. Gently clean top membrane with cotton swab
2. Add 2.5 mL of cold methanol to new wells and let inserts sit for 10 minutes
3. Wash with PBS
4. Add 2 mL of crystal violet to new wells and let inserts sit for 10 minutes
5. Rise with diH2O until most of crystal violet is out
6. Take pictures of membranes after dried out – 20X on “H”

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