

**CIRCULATING EXOSOMES SECRETED BY OSTEOSARCOMA CELLS  
ARE POTENTIAL BIOMARKERS FOR METASTASIS**

by

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## **ABSTRACT**

Osteosarcoma is a primary malignant bone tumor found in children and is the third most common cancer in children and adolescents. Upon diagnosis, patients undergo surgery and aggressive chemotherapy treatments resulting in a high prognosis for survival. However, once metastasis occurs, primarily to the lung, prognosis is poor. Recent chemotherapy clinical trials have provided few successes, leading researchers to look into other non-invasive biomarkers for therapeutic development. One way to obtain biomarkers is through the use of liquid biopsies which are body fluids obtained from cancer patients at diagnosis. These liquid biopsies provide a way to analyze what tumors are secreting into the bloodstream. The contents of these biopsies give insight into cancer progression and provide multiple avenues for diagnostic marker research.

Exosomes are small extracellular vesicles that contain a multitude of biomolecules varying from proteins, DNA, RNA (e.g., mRNA and miRNAs), lipids, and metabolites. These nanoparticles are released by an origin cell into the bloodstream with content that is reflective of its origin. Exosomes are secreted by all cells but they are secreted at a higher rate from cancer cells compared to normal cells. Therefore, there is a higher concentration of tumor-derived exosomes in cancer patients that can be utilized as a way to track metastasis. In order to track metastasis in exosomes, a specific biomolecule in their cargo needs to be used as a cancer-specific target for tracking and screening throughout the body. Based on previous research, our lab focuses on p27/Kip1 protein as a potential target in osteosarcoma-derived exosomes.

p27 is a tumor suppressor involved in cell cycle regulation when localized to the nucleus, but upon localization out into the cytoplasm it facilitates oncogenic activity. Cytoplasmic p27 acts as an oncoprotein and is involved in many cellular processes including cytoskeletal dynamics and cell migration, therefore it is associated with tumorigenesis and disease progression. With this key characteristic of osteosarcoma, further research is needed to assess if p27 in exosome cargo acts as a mechanism of metastasis. I hypothesize that p27 protein is secreted in exosomes by osteosarcoma cells and facilitates metastasis to the lung.

In this study, I examined the expression of p27 in osteosarcoma-derived exosomes and the uptake of exosomes by human lung fibroblasts. I investigated p27 expression in osteosarcoma-derived exosomes from multiple osteosarcoma cell lines compared to normal cells using immunoblot analysis. I found that exosomes secreted by human osteoblasts and human lung fibroblasts did not contain p27 in their cargo, but all three osteosarcoma cell lines (143B, HOS, and MG63) did. After confirmation that p27 in exosomes is osteosarcoma specific, I evaluated the uptake of exosomes by human lung fibroblast cells. Using immunofluorescence and immunoblot analysis, I found that exosomes are taken up by lung fibroblasts thus influencing both the content and proliferation of the lung fibroblast cells. In summary, this data provides insight into the role of osteosarcoma-derived exosomes in metastasis and provide a potential source of biomarkers for therapeutic development.

## **Chapter 1**

### **INTRODUCTION**

#### **1.1 Osteosarcoma**

Osteosarcoma is a primary malignant bone tumor found in children with a peak incidence in the second decade of life (Misaghi et al., 2018). It is the third most common malignancy in children and adolescents. This is a rare sarcoma that is thought to be associated with mesenchymal cells that undergo abnormal differentiation to osteoblasts, resulting in heterogeneous tumors. These sarcoma tumors are typically localized in the long bones (e.g., femur, humerus, and tibia) due to the acute linear bone growth during puberty (Czarnecka et al., 2020). Upon diagnosis with osteosarcoma, survival rates range from 70-80%. Most patients present with localized disease, but upon the diagnosis of metastatic disease, most commonly to the lung and other bones, survival rates decrease significantly to 20-30% (Czarnecka et al., 2020). Common subtypes of conventional osteosarcoma tumors are classified as osteoblastic, chondroblastic, and fibroblastic with osteoblastic considered to be the most common of the three (X. Zhao et al., 2021). Development of tumors can be linked to chromosomal abnormalities and/or genetic mutations. Chromosomal abnormalities consist of numerical and structural abnormalities. Numerical chromosomal abnormalities are the gain or loss of a chromosome and approximately 90% of cancers are linked to the gain or loss of at least one chromosome (Kou et al., 2020). Structural abnormalities are caused by DNA damage in the chromosomes and are typically classified as deletions, amplifications, and unbalanced translocations (Kou et al.,

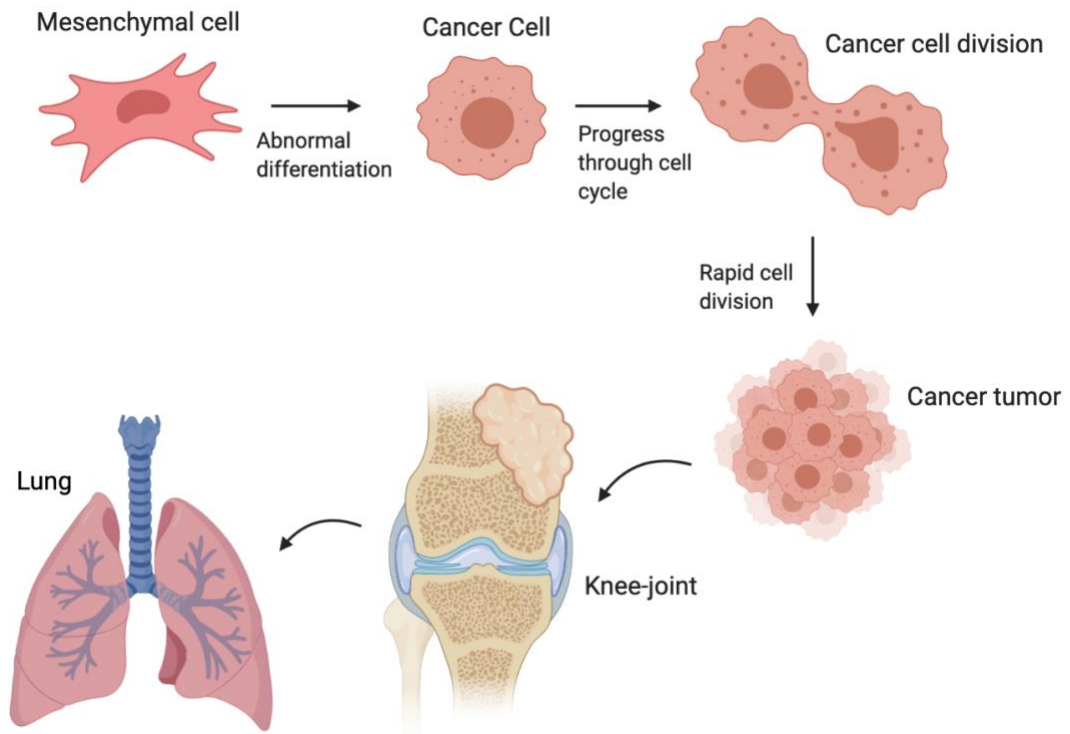
2020). Osteosarcoma is characterized by its chromosomal instability (CIN), a numerical chromosomal abnormality, 70% of osteosarcoma tumors have been linked to these abnormalities (Tang et al., 2008). The high variability of genetic modifications is often characterized by the deletion and/or amplification of certain chromosome regions (Table 1.1). Osteosarcoma tumors are considered to be heterogeneous indicating that tumor development is not linked to specific genetic factors, but researchers found that approximately 28% of patients with osteosarcoma had a pathogenic mutation in cancer-susceptibility genes (Mirabello et al., 2020) (Table 2.1).

Genomic region	Event	Affected Gene
3q13.31	Deletion	<i>LSAMP</i>
6p12-p21	Gain	<i>RUNX2, CDC5L</i>
6p12-p21	Amplification	<i>VEGFA, PIM1</i>
6p22.3	Gain	<i>E2F3</i>
7p21	Deletion	<i>TWIST</i>
7p31	Amplification	<i>MET</i>
8q24.21	Amplification	<i>MYC</i>
9p21	Deletion	<i>p16/INK4A, p14/ARF, p15/INK4B</i>
10q26	Loss of heterogeneity	<i>BUB3, FGFR2</i>
12q13	Amplification	<i>PRIM1</i>
12q15	Amplification	<i>MDM2</i>
16q23.1-q23.2	Deletion	<i>WWOX</i>
17p11.2-p12	Amplification	<i>COPS3, PMP22, MAPK7</i>

**Table 1.1 Chromosomal abnormalities involved in osteosarcoma development.** Known chromosomal abnormalities that result in osteosarcoma development in patients (Mirabello et al., 2020).

Gene mutations	Gene Description	Inheritance pattern	Role
TP53	Tumor protein p53	Dominant	Transcription
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Dominant	Cell cycle
MEN1	Menin 1	Dominant	Transcription
VHL	Von Hippel-Lindau tumor suppressor	Dominant	Structure
POT1	Protections of telomeres 1	Dominant	Telomeric binding
APC	APC regulator of WNT signaling pathway	Dominant	WNT signaling
MSH2	MutS homolog 2	Dominant	DNA mismatch repair
RB1	RB transcriptional corepressor 1	Dominant	Transcription
RECQL4	RECQ PROTEIN-LIKE 4	Recessive	DNA helicase
RPL35A	Ribosomal protein L35A	Dominant	Ribosomal protein
RPL5	Ribosomal protein L5	Dominant	Ribosomal protein
RPS19	Ribosomal protein S19	Dominant	Ribosomal protein
RPS7	Ribosomal protein S7	Dominant	Ribosomal protein

**Table 1.2 Gene mutations involved in osteosarcoma development.** Known gene mutations that result in osteosarcoma development in patients (Mirabello et al., 2020).



**Figure 1.1 Osteosarcoma tumor development and metastasis to the lung.**

Formation of an osteosarcoma tumor is thought to be associated with a mesenchymal stem cell, commonly found in bone marrow, undergoing abnormal differentiation within the growth plate of long bones. This abnormal differentiation results in a cancer cell which then progresses through the cell cycle, resulting in increased cell proliferation. The most common tumor site in patients is in the metaphysis of the long bones. Once an osteosarcoma tumor develops, metastasis can occur most frequently in the lungs. Figure is generated with BioRender.

## **1.2 Diagnosis**

Clinical onset of osteosarcoma in patients is commonly characterized by localized pain in the long bones (e.g., femur or humerus), swelling, limping (in the legs), and/or joint pain. To diagnose a patient, imaging techniques are used to detect and clinically identify osteosarcoma tumors: X-ray, Computed Tomography (CT), and Magnetic Resonance Imaging (MRI). Performing x-rays on a patient provides a comprehensive way to display the location and size of the tumor in a patient while CT scans are used for bone scanning to visualize the full extent of the invasion of cancer both inside and outside of the tumor site (X. Zhao et al., 2021). MRI is used as well to determine the stage of cancer and help to establish preoperative planning for a patient. This method is more beneficial compared to x-rays and CT scans because it helps identify the range of the tumors found in the intramedullary space of the bone, the size of soft tissue masses, and all of the surrounding tissues (X. Zhao et al., 2021). While these diagnostic methods are the standard for diagnosis, there are no laboratory tests that are diagnostic for osteosarcoma specifically

## **1.3 Metastasis**

Metastasis is the spread of cancer cells from the primary tumor site to a secondary site in the body. For cancer to metastasize to another site in the body it must progress through a specific series of steps, referred to as the metastatic cascade. This stepwise process begins with the localized detachment of cells from the primary tumor, invasion and migration into the tumor microenvironment (TME), diffusion of malignant cells out into the surrounding extracellular matrix followed by intravasation, the invasion of cancer cells into blood or lymphatic vessels. Cancer cells commonly use the hematogenous route for circulation because it provides higher blood vessel

accessibility which assists in tumor angiogenesis (Serenio et al., 2020). The metastatic cascade is a generalized sequence that cancer would follow, and in the case of osteosarcoma, metastasis is the most frequent cause of death among patients with osteosarcoma. To gain further insight into cancer metastasis researchers investigate cell proliferation pathways such as the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway. The MAPK pathway is involved in many molecular processes such as cell proliferation, cell survival, and tumor progression (Sinkala et al., 2021). The PI3K/AKT pathway is another cell proliferation pathway that has been shown to promote tumor cell proliferation, cell invasion, and inhibits apoptosis (Liu et al., 2020). In addition to proliferative pathways, metastatic genes such as vimentin, SMAD family member 4 (SMAD4), Snail2/SLUG, and N-cadherin are involved in disease progression and are studied as possible prognostic markers for osteosarcoma metastasis. Vimentin is mesenchymal-specific intermediate filament protein that is used as a canonical marker and is expressed during the epithelial-mesenchymal transition (EMT) (Dmello et al., 2017). SMAD4 is a mediator of the transforming growth factor  $\beta$  (TGF- $\beta$ ) signaling pathway that is involved in multiple cellular processes including proliferation, migration, apoptosis, cancer initiation, and progression (M. Zhao et al., 2018). Snail2 is a zinc finger transcription factor involved in EMT and has been shown to promote tumor invasion (Hu et al., 2018; Villarejo et al., 2015) and N-cadherin is a calcium-dependent adhesion molecule that is involved in cancer migration and invasion (Zannettino et al., 2018). In order to detect early pulmonary metastasis of osteosarcoma and improve survival, it is important to further explore more effective prognostic biomarkers and therapeutic targets.

## 1.4 Treatment

Treatment of osteosarcoma involves the use of aggressive chemotherapy and surgery. Before the use of chemotherapy, treatment solely consisted of limb amputation until the introduction of chemotherapy in the 1970s. The current standard treatment regime consists of neoadjuvant chemotherapy and surgery, followed by adjuvant chemotherapy. Surgery in the context of osteosarcoma can be classified into two types: amputation and limb salvage. Limb salvage surgery is the process where surgeons restore bone and joint function after extensive tumor resections within the affected limbs. While both surgical methods can be used in the treatment regime, limb salvage patients have shown a higher 5-year survival rate compared to amputation patients (X. Zhao et al., 2021). In order to successfully treat osteosarcoma with chemotherapy, the only agents that have been shown to be most effective against tumors are **M**ethotrexate, **A**driamycin (doxorubicin), and **C**isplatin or MAP agents. Methotrexate is classified as an antimetabolite, that interferes with the S-phase of the cell cycle and directly impedes DNA replication (Lilienthal & Herold, 2020; Mills et al., 2018). Adriamycin is classified as an anthracycline, it intercalates into the DNA of a cell and disrupts the topoisomerase-II-mediated DNA repair mechanism (Thorn et al., 2011). Cisplatin is an alkylating agent that causes DNA damage ultimately leading to apoptosis or programmed cell death (Lilienthal & Herold, 2020). Over the past three decades, neoadjuvant and adjuvant chemotherapy have effectively improved safe limb-sparing resections, and disease-free and overall survival rates have increased. Long-term survival and cure rates have risen to 60-80% in patients with localized disease. Since the introduction of these chemotherapy agents, there have been no significant advances in treatment (Misaghi et al., 2018). Once metastatic tumors

develop in a patient, commonly within 5 years after remission, the only course of action is to treat with salvage chemotherapy and remove metastatic lesions surgically.

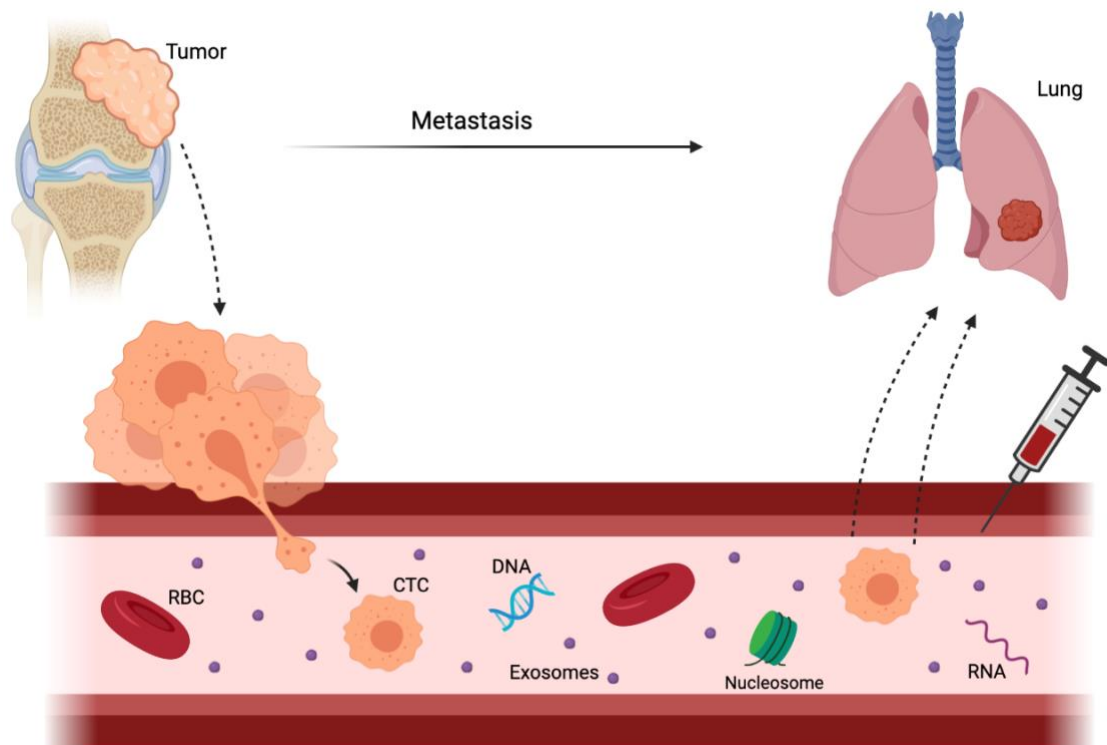
Although chemotherapy is effective in treating cancer there are resulting complications following chemotherapy treatments. Common side effects during treatment include hair loss, vomiting, fatigue, mouth sores, etc. While these are routine symptoms during treatment, there are long term side effects that manifest months later (e.g., cardiomyopathy, renal disease, nerve damage, infertility, etc.). Using chemotherapy is effective in treating primary tumors but once relapse occurs successful treatment using chemotherapy is diminished. When a patient relapses, they must undergo multiple surgeries to resect metastatic tumors and continue chemotherapy treatments. In many cases, metastatic tumors do not respond well to chemotherapy, this is due to metastatic tumors frequently having chemotherapy resistance. Due to the nature of these metastatic tumors, drug development is the keyway to ensure effective treatment and patient survival. Current clinical trials for osteosarcoma drug development have proved minimal success (e.g., cabozantinib and sorafenib). Cabozantinib is a vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor and showed promising effects in individuals with advanced osteosarcoma and current clinical trials are ongoing (Czarnecka et al., 2020). Sorafenib is a tyrosine kinase inhibitor that when used in clinical trials proved to be less effective but when combined with other molecular targeted agents it could be promising but requires further studies (Czarnecka et al., 2020). There have been many clinical trials for the treatment of osteosarcoma, but patient outcomes remain limited leaving therapeutic success just as elusive as before. Thus, further investigation is

needed to identify diagnostic and therapeutic strategies using non-invasive methods including biomarkers in liquid biopsies.

### **1.5 Biomarkers and biopsies**

A keyway researchers and doctors monitor and develop our understanding of cancer is through the use of liquid biopsies obtained from cancer patients. Liquid biopsy tests can be performed on body fluids such as blood, urine, saliva, and cerebrospinal fluid. The use of these biopsies whether it is obtained at diagnosis, resection, or relapse is considered the “gold standard” for therapeutic planning and diagnosis (Weiser et al., 2019). The contents of these liquid biopsies provide insight into what cancer tumors are secreting into the bloodstream. In turn, this gives researchers possible avenues for identification of diagnostic markers, drug targets, and understanding mechanisms of cancer development and progression. Some circulating analytes found in liquid biopsies are circulating tumor cells (CTCs), cell-free DNA, cell-free RNA, macromolecular structures, and noncoding RNAs (ncRNAs) (Weiser et al., 2019). CTCs are shed from the tumor directly into the bloodstream and as a result, have genetic compositions similar to the tumor cells. Since their discovery, CTCs have been established across an abundance of malignancies varying from patients with metastatic lung, breast, and prostate cancer (Weiser et al., 2019). Some of the macromolecular structures that are released into the bloodstream are nucleosomes, coiled DNA surrounding histones, and small extracellular vesicles called exosomes whose cargo varies from proteins, RNA, lipids, and metabolites (Hoshino et al., 2015). Exosomes have been shown to be a promising focus for liquid biopsies because cancer cells secrete exosomes at a higher rate compared to normal cells

making tumor-derived exosomes (TEX) a potential target in diagnostics and prognosis (Sinha et al., 2021).



**Figure 1.2 Content of liquid biopsies from osteosarcoma patients.** Liquid biopsies from cancer patients contain CTCs, DNA, RNA, nucleosomes, and exosomes. Figure is generated with BioRender.

## 1.6 Exosomes

Extracellular vesicles are lipid-bound vesicles secreted by cells into the extracellular space and can be broken down into three subtypes: microvesicles, exosomes, and apoptotic bodies (Doyle & Wang, 2019). These vesicles are classified based on origin, size, content, and specific markers. Microvesicles have a size range of 100nm-1 $\mu$ m and form by direct outward budding of the plasma membrane of the cell (Doyle & Wang, 2019). These small vesicles are involved in cell-cell communication, more specifically communication between both local and distant cells (Doyle & Wang, 2019). Exosomes are nano-vesicles secreted by all cells and have a size range of 30-150nm. In contrast to microvesicles, these vesicles are formed by the inward budding of the membrane and as a result have a lipid membrane (Chicón-Bosch & Tirado, 2020). The overall postulated function of these vesicles is that they are involved in cell-cell communication, cell maintenance, and tumor progression. Apoptotic bodies are different from both microvesicles and exosomes in that they are not released by healthy cells. Apoptotic bodies are vesicles released by cells that are undergoing apoptosis or programmed cell death. During this process the cell begins to shrink, chromatin condenses, nuclear fragmentation occurs, and lastly, the plasma membrane begins to bleb (Mehrbod et al., 2019). These vesicles range in size from 50nm to 5  $\mu$ m and as a result have different proteomic profiles compared to both microvesicles and exosomes (Doyle & Wang, 2019).

Of the three subtypes of extracellular vesicles, the one of particular interest for liquid biopsy studies is exosomes. Exosomes contain many cellular components in their cargo including DNA, RNA, metabolites, and lipids that are transported to recipient cells that could potentially alter biological processes (Kalluri & LeBleu, 2020). The exosome-mediated responses in recipient cells could be altering cells in a

way that could promote disease development such as cancer progression or disease prevention via an immune response. Exosomes are released from the donor cell through the invagination of the plasma membrane. This invagination causes the formation of endosomes which mature to form multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs), ILVs are ultimately shed as exosomes (Kalluri & LeBleu, 2020; Sinha et al., 2021). These lipid-bound vesicles have a membrane rich in tetraspanins (CD9, CD81, and CD63), integrins, and adhesion molecules that assist in receptor-ligand binding with recipient cells. Once an exosome is released into the extracellular matrix, exosomes participate in receptor-ligand binding, exosomes are then internalized into the recipient cells by (1) direct fusion with the plasma membrane (2) phagocytosis (3) macropinocytosis (4) clathrin/caveolin-mediated endocytosis and (5) uptake via lipid rafts (Sinha et al., 2021). These internalizations trigger a cellular response, but it is difficult to know which model of uptake results in a specific functional outcome of the cargo, degradation, or localization, once it is incorporated in the recipient (Kalluri & LeBleu, 2020).

Once an exosome is taken up by a recipient cell there are many possible cellular responses that could occur after uptake. After an exosome is taken up, exosomes can fuse with the lysosome leading to degradation or exosomes can fuse with pre-existing early sorting endosomes (Kalluri & LeBleu, 2020). After an exosome fuses with an early sorting endosome, the exosomes are disintegrated and their contents are either released into the cytoplasm or the endoplasmic reticulum (Kalluri & LeBleu, 2020). A promising area of research is that exosomes contain cargo that is reflective of the original donor cell for cancer cells that secrete exosomes

at a higher rate, this could indicate roles that exosomes play in tumorigenesis and metastasis.

### **1.7 p27/Kinase protein inhibitor 1 (p27/Kip1)**

p27/Kip1 (p27) protein has been shown to inhibit cyclin-dependent kinase (CDK)2-cyclin E and CDK4-cyclin D activity in the G1 phase of the cell cycle, ultimately interrupting transition into the S-phase (Currier et al., 2019). When conditions are favorable for the cell to undergo proliferation, p27 levels begin to decrease and the cell undergoes the G<sub>1</sub>/S transition. While this is the established function, localization of p27 from the nucleus into the cytoplasm in cancer is associated with additional p27 functions that suggests nuclear p27 functions as a tumor suppressor, while cytoplasmic p27 functions as an oncoprotein. While nuclear p27 is undetectable in multiple cancers (e.g., lung, breast, and colon cancer), increased cytoplasmic p27 in the cell correlates with tumor aggressiveness ultimately leading to poor patient outcomes (Sharma & Pledger, 2016). Cytoplasmic p27 has pivotal roles in multiple cellular processes such as cytoskeletal dynamics and cell migration, and as a result, it is commonly associated with tumorigenesis and disease progression. Cytoplasmic p27 is involved with cytoskeletal dynamics due to its association with stathmin (STMN1), a microtubule (MT) destabilizing protein prominent during mitosis, further conferring oncogenic roles to cytoplasmic p27 (Atweh & Iancu-Rubin, 2014). STMN1 is a highly conserved cytoplasmic protein that is overexpressed in multiple malignancies and metastasis (Atweh & Iancu-Rubin, 2014). Previous research in our lab on p27 in osteosarcoma found that p27 primarily localizes into the cytoplasm but remained localized in the nucleus within normal cells (Currier et al.,

2019). Further research on p27 in osteosarcoma could not only provide insight into an elusive disease but could be used as a biomarker to track the disease.

## **1.8 Hypothesis and Experimental aims**

Hypothesis: p27 protein secreted in exosomes by osteosarcoma cells may facilitate metastasis to the lung.

Aim 1: Isolate tumor derived exosomes from osteosarcoma cells and characterize p27 expression.

Aim 2: Evaluate the uptake of osteosarcoma derived exosome by human lung fibroblast cells.

## **Chapter 2**

### **MATERIALS & METHODS**

#### **2.1 Tissue Culture**

Human osteosarcoma cell line (143B), human lung fibroblast (HFL1), and human osteoblasts (CRL-11372) were purchased from ATCC (VA). The growth media used were Minimal Essential Medium (MEM) for 143B cells, and F-12K for HFL1 fibroblasts and DMEM without phenol red for CRL-1132 osteoblast cells. MEM and F-12K media were supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (P/S) and DMEM was supplemented with 10% FBS and G418. Cells were maintained at 37° C in humidified incubators.

#### **2.2 List of antibodies**

- Alexa Fluor 488 goat anti-rabbit IgG (Life Sciences, CA)
- Alix (E6P9B) Rabbit mAb (Cell Signaling Technologies, MA)
- anti-rabbit IgG HRP-linked antibody (Cell Signaling Technologies, MA)
- Calnexin (C5C9) Rabbit mAb (Cell Signaling Technologies, MA)
- CD9 (D801A) Rabbit mAb (Cell Signaling Technologies, MA)
- Flotillin-1 (D2V7J) XP (R) Rabbit mAb (Cell Signaling Technologies, MA)
- GAPDH (14C10) Rabbit mAb (Cell Signaling Technologies, MA)
- Integrin-beta1 Rabbit Ab (Cell Signaling Technologies, MA)

- N-cadherin (D4R1H) Rabbit mAb (Cell Signaling Technologies, MA)
- p27 Kip1 (D69C12) XP (R) Rabbit mAb (Cell Signaling Technologies, MA)
- P-p44/42 MAPK (T202/Y204) Rabbit Ab (Cell Signaling Technologies, MA)
- Slug (C19G7) Rabbit mAb (Cell Signaling Technologies, MA)

### **2.3 Ultracentrifugation**

CRL-11372, HFL1 and 143B cells were grown in T75 flasks containing 10 ml of exosome free growth media. When cells were between 75-90% confluency, media was collected for isolation of exosomes. Media containing exosomes were centrifuged at 2,000g at 4°C for 20 minutes and the pellet containing cellular debris was discarded. The resulting supernatant was centrifuged using the Optima L-90K Ultracentrifuge (Beckman Coulter, CA) at 10,000 rpm at 4°C for 30 minutes and the pellet containing larger extracellular vesicles was discarded. The supernatant was centrifuged at 24,000 rpm at 4°C for 4 hours. The supernatant was discarded and the pellet containing exosomes was resuspended using 100 µl of phosphate-buffer saline (PBS), pH 7.4 and stored at -20 °C.

### **2.4 Nanoparticle Tracking Analysis (NTA)**

The NanoSight NS300 (Malvern Panalytical, UK) was utilized for particle sizing of exosome nanoparticles according to the manufacturer's instructions. Camera levels and sample concentration were adjusted to yield 25-35 particles per frame during capture. Results are reported as images.

## **2.5 Bicinchoninic Acid (BCA) protein assay**

Protein concentrations of whole cell lysates and exosome lysates were determined with BCA assay using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, CA). Bovine serum albumin (BSA) standards (Thermo Scientific, IL) were used to prepare a standard curve. Triplicate volumes of each standard were pipetted into a 96-well plate. Cell lysates were diluted (1:20) in radioimmunoprecipitation (RIPA) buffer and triplicate volumes were pipetted into the plate. Exosome lysates were not diluted and triplicates volumes were pipetted into the plate. Solution of BCA protein assay reagent A and reagent B (Thermo Scientific, IL) was prepared and A/B was added per well for the triplicates for the standards and samples. The plate was incubated for 30 minutes at 37°C. After incubation absorbance at 562 nm was read on the Victor 5 Plate Reader (PerkinElmer, MA). Resulting data was analyzed to determine protein concentration.

## **2.6 Western Blot**

Cell lysates: HFL1 cells were grown to 75% confluency in T75 flasks and treated with  $1 \times 10^{12}$  exosomes for 48 hours. After cells were washed with PBST and lysed in RIPA buffer (Invitrogen, CA) containing protease and phosphate inhibitors (Thermo Fisher Scientific, CA). Lysates were sonicated and cleared of cellular debris via centrifugation. Exosome lysates: After exosomes were isolated from growth media via ultracentrifugation (Section 2.2), exosomes were lysed in 10% sodium dodecyl sulfate (SDS). Cell lysates and exosome lysates were used to determine protein concentration using the BCA assay (Invitrogen, CA). Samples containing 25  $\mu$ g of protein were denatured with Lamelli 4x sample buffer (Bio-Rad, CA) containing 5%  $\beta$ -mercaptoethanol (Thermo Fisher Scientific, CA). Samples were heated at 100°C for

5 minutes. Proteins were resolved by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS/PAGE) on 10% Tris-glycine gels (Invitrogen, CA), transferred to a nitrocellulose membrane and blocked for 30 minutes with 5% milk in PBS containing 0.03% Tween-20 (PBST) at room temperature. Membranes were then incubated overnight at 4°C in primary antibody diluted (1:1,000) in PBST containing 5% BSA. After incubation membranes were washed in PBST and incubated for 1 hour at room temperature in horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, MA) in 5% milk then washed with PBST. Antibodies against GAPDH was used as the endogenous control for cell lysates. Antibodies against ALIX and Flotillin were used as the endogenous control for exosome lysates. Band intensity was calculated using Image J (U.S. National Institutes of Health, MD). T-test analysis was performed to calculate statistical significance.

## **2.7 Immunostaining and Immunofluorescence**

HFL1 cells were grown on glass coverslips (pre-coated with 0.01% poly-L-lysine (Gibco, MA)) in 12-well cell-culture plates and were incubated with 143B exosomes that were immunostained with anti-p27 rabbit monoclonal or anti-CD9 rabbit monoclonal antibody overnight at 4°C, followed by an Alexa 488 (A11034) (Life Sciences, CA) anti-rabbit conjugated secondary antibody (Thermo Fisher Scientific, IL) for 1 hour. Labelled exosomes were diluted with PBS, transferred to a Vivaspin spin column and centrifuged at 12,000g for 4 minutes at 4°C. Exosomes were washed and resuspended with PBS. Cells were incubated with labelled exosomes for 4 hours at 37°C, washed with PBST, then fixed with 4% paraformaldehyde. Nuclei were stained with Hoechst dye (Thermo Fisher Scientific, IL) for 10 minutes at room temperature (Invitrogen, CA). Coverslips were mounted onto slides. Samples were

digitally imaged on a Zeiss LSM-confocal microscope with AiryScan (Carl Zeiss Microscopy, LLC, Thornwood, NY) controlled by Zen software.

## **2.8 siRNA transfection**

143B cells were cultured in T75 flasks containing MEM media at 37°C, then treated with 1 mL of Opti-MEM containing 10 nmol of *CDKN1B* siRNA (Dharmacon, CO) and of Lipofectamine RNAiMAX reagent (Invitrogen, CA). 10 nmol of *CDKN1B* siRNA was diluted in Opti-MEM and mixed with Lipofectamine diluted in Opti-MEM. The mixture was incubated for 20 minutes prior to treatment of 143B cells. After adding Opti-MEM containing *CDKN1B* siRNA and Lipofectamine, flasks were incubated at 37°C in humidified incubators for 48 hours. Following incubation, media was obtained for exosome isolation; cells were trypsinized and cell lysates were prepared for protein analysis.

## **2.9 Exosome Internalization assay**

a. Fluorescent labeling of exosomes using PKH67 Green Fluorescent Cell Linker Mini Kit (Sigma Aldrich, MO): Purified 143B exosomes were resuspended in PBS after isolation via ultracentrifugation (Section 2.2). Unlabeled control exosomes and PKH67-labelled exosomes were pelleted by centrifugation at 10,000 g for 5 minutes at 21°C. Supernatant was removed and Diluent C (Sigma Aldrich, MO) was added to control and PKH67. For exosome labeling, PKH67 fluorescent dye linker (Sigma Aldrich, MO) was added to PKH67-labelled sample only. Tubes containing exosomes were incubated at room temperature on a shaker for 30 minutes. After incubation labeled exosomes were pelleted and washed in PBS buffer. b. Treatment of HFL1 cells: HFL1 cells were seeded at  $2 \times 10^5$  cells/well into 12-well plates in growth media

and grown overnight in humidified incubators at 37°C. Cell growth media was added to each tube containing exosomes then introduced to seeded HFL1 cells in a 12-well plate. Exosomes were incubated with lung fibroblast cells for 4 hours at 37°C. After incubation media was removed cells were washed with PBS. Cells were fixed using 4% paraformaldehyde and exosome internalization was visualized by confocal microscopy (Zeiss LSM-confocal microscope with AiryScan controlled by Zen software (Carl Zeiss Microscopy, LLC, Thornwood, NY)).

### **2.10 Cell viability assay**

HFL1 and CRL-11372 cells were seeded in separate 96-well plates at  $2 \times 10^3$  cells/well and grown overnight in humidified incubators at 37°C. 143B exosomes were introduced to HFL1 and CRL-11372 cells and incubated for 48 hours. After incubation was complete, viable cells were measured with Cell Titer Blue assay (Promega, WI) reagent. After incubation for 4 hours at 37°C, the absorbance was read at 570 nm using a Victor 5 Plate Reader (PerkinElmer, MA) at excitation/emission wavelengths of 550/590 nm. Data are represented as the mean of 3 measurements  $\pm$  SD. Cell viability and t-test statistical analysis were conducted with Excel.

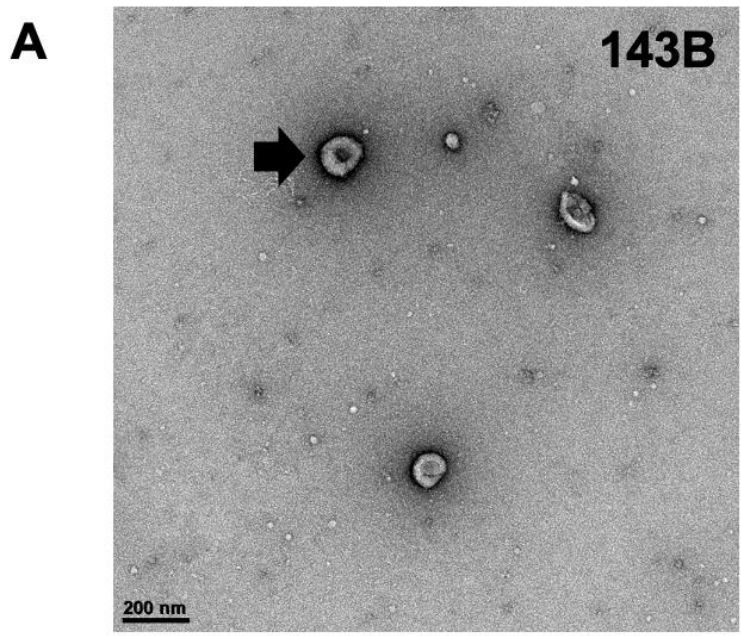
## Chapter 3

### RESULTS

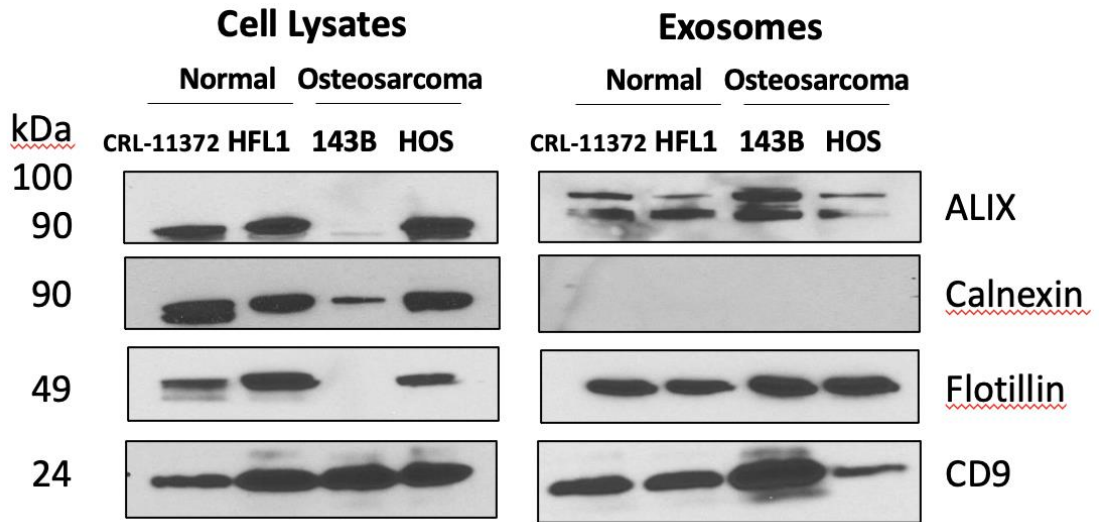
#### 3.1 Characterization of osteosarcoma derived exosomes

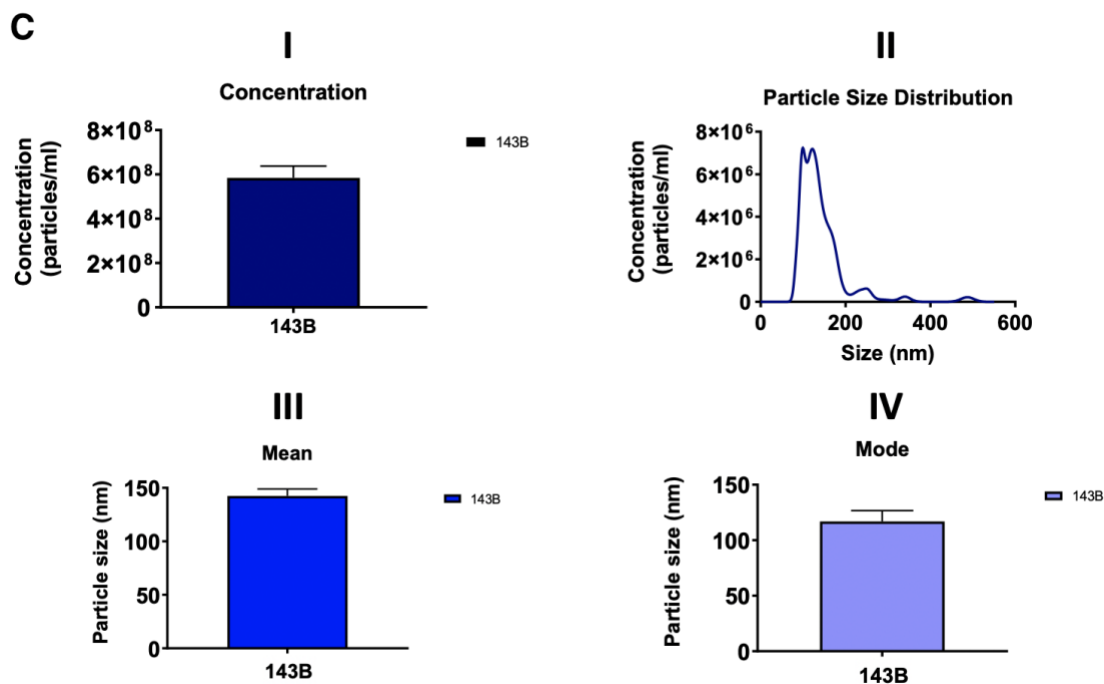
The use of extracellular vesicles requires adherence to the “minimal information for studies of extracellular vesicles (MISEV)” protocol to confirm the presence and purity of extracellular vesicles in research (Théry et al., 2018). The requirements of MISEV are quantification, size determination, analysis of biophysical features, presence of membrane-bound cytosolic compartment, purification, compartment of origin, and functional proteins. Exosomes were isolated from human osteosarcoma cell line 143B via ultracentrifugation (Section 2.2). Whole exosomes were analyzed to determine purity and particle size by Transmission Electron Microscopy (TEM). Figure 3.1A is a TEM image of 143B-derived exosomes. This TEM image shows the presence of a pure population of 143B-derived cup-shaped exosomes based on their size (< 200 nm) which are indicated by the black arrow. Figure 3.1B is western blot analysis of cell lysates and secreted exosomes of normal cells (CRL-11372 and HFL1) and of osteosarcoma cells (143B and HOS). These immunoblots were treated with antibodies against ALG2 Interacting Protein X (Alix), flotillin, CD9, and calnexin. Alix, flotillin, and CD9 were used as positive markers while calnexin was used as a negative marker. Alix is involved in the endosomal sorting complex required for transport (ESCRT) and is commonly used as an endosomal marker due to its involvement in the biogenesis of exosomes (Sun et al., 2015). Flotillin is an integral membrane protein involved in the formation of non-

caveolar microdomains and functions as a marker for lipid rafts such as exosomes (Meister & Tikkanen, 2014). CD9 is a tetraspanin, a transmembrane protein, found in exosome membranes and are organized into tetraspanin-enriched domains (TEMs) (Whiteside, 2016). These abundant cell-surface proteins play an important role in exosome biogenesis and are popularly used as exosome markers. Calnexin is a type I integral endoplasmic reticulum (ER) membrane protein that acts as a molecular chaperone and is present in cells but not in exosomes (Lee et al., 2015). Figure 3.1C is Nanoparticle tracking analysis of whole 143B exosomes. Graph I is the concentration of exosome particles/ml, in our sample the concentration was  $5.84 \times 10^8$  particles/ml. Graph II is the particle size distribution within the sample, indicating that the particles in our sample were a size of less than 200 nm. Graph III is the mean particle size in our sample, which was 142.4 nm, this data confirmed the particles were within the expected size range of exosomes, 30 - 150 nm. Graph IV is the mode particle size of our sample at 116.9 nm. Taken together, Figure 3.1 confirms that our sample is of a pure population of exosomes that fall within the proper particle size range of 30 - 150 nm.



**B**



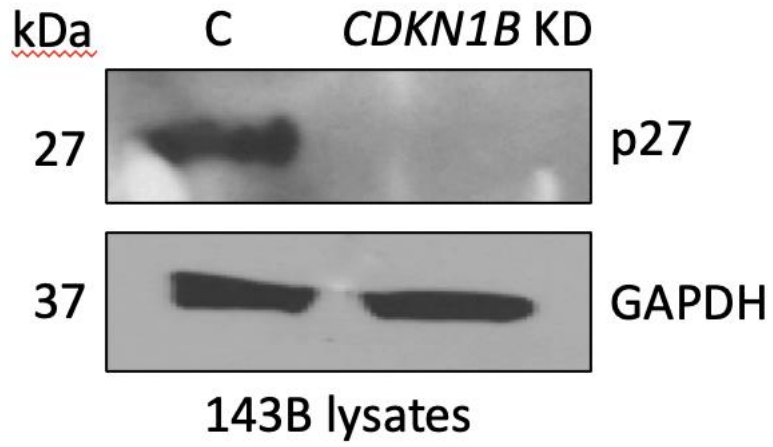


**Figure 3.1 Analysis of exosomes derived from 143B cells.** (A) Exosomes were purified from growth media of 143B cells using ultracentrifugation. Sample was visualized using Transition Electron Microscopy. Black arrows indicate cup-shaped pure exosomes. (B) Cell lysates and exosome lysates were extracted from human osteoblasts (CRL-11372), human lung fibroblasts (HFL1) and 143B, HOS human osteosarcoma cell lines. Lysates were examined by immunoblot analysis. ALIX, Flotillin, and CD9 were positive exosome markers. Calnexin was the negative exosome marker. (C) Nanoparticle tracking analysis of whole 143B exosomes in PBST (Graph I) particle concentration within the sample in particles/mL (Graph II) particle size distribution of the sample in nm. (Graph III) analysis of the mean particle size in the sample (Graph IV) mode particle size in the 143B-derived exosomes. Error bars indicate mean value  $\pm$  SD.

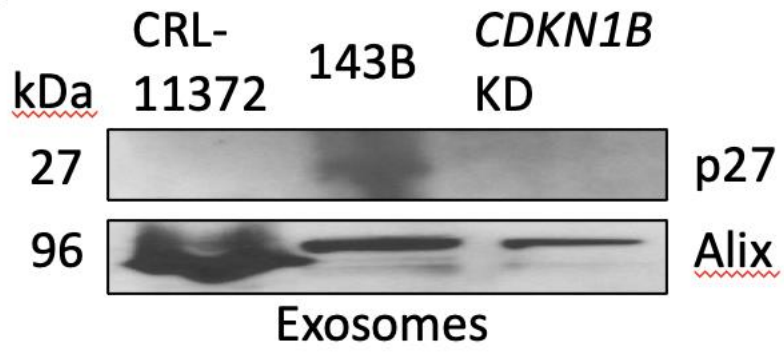
### **3.2 *CDKN1B* siRNA knockdown inhibits p27 expression in human osteosarcoma exosomes**

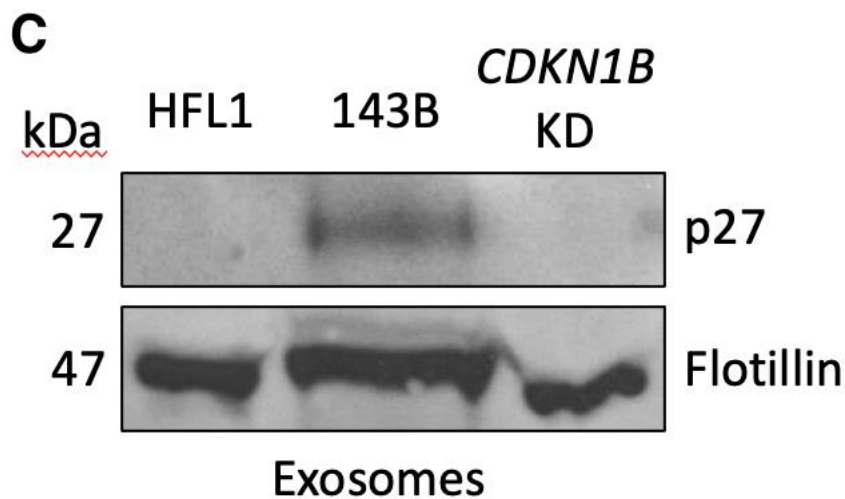
Previous research in our lab on p27 in osteosarcoma found that p27 is highly expressed in the cytoplasm as well as in metastatic tumors (Currier et al., 2019). Due to this, I investigated the expression of p27 in human osteosarcoma-derived exosomes as a target. To further confirm the presence of p27 in osteosarcoma exosome cargo, I cultured a second flask of osteosarcoma cells to undergo a *CDKN1B* siRNA knockdown to inhibit p27 expression. I cultured two normal cell lines, CRL-11372 and HFL1, and 143B cells. I also performed p27 knockdown on 143B cells (Section 2.5) using *CDKN1B* siRNA. Figure 3.2A shows the transient knockdown of p27 in 143B cells by *CDKN1B* siRNA compared with untransfected cells as a control. Next, I isolated exosomes from the respective cell growth media (Section 2.2) to analyze p27 protein expression in exosomes. Figure 3.2B and C immunoblots show p27 is solely expressed in osteosarcoma-derived exosomes but not in control exosomes (CRL-11372 and HFL1) or following siRNA mediated p27 mediated p27 knockdown in 143B cells, establishing that p27 in osteosarcoma cells have abnormal expression and is secreted into exosomes. Alix and Flotillin were used as exosome markers to confirm exosome analysis

**A**



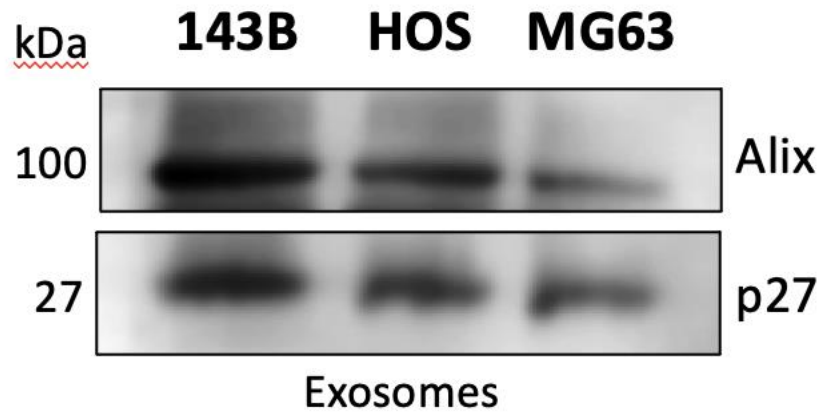
**B**





**Figure 3.2 p27 expression is inhibited in exosomes following siRNA mediated knockdown.** (A) Lysed 143B cells, C are normal 143B cells without *CDKN1B* knockdown and *CDKN1B* KD are 143B cells with the siRNA knockdown, were analyzed by immunoblotting with anti-p27 antibody. GAPDH was the endogenous loading control. (B) Lysed exosomes were analyzed by immunoblotting with anti-p27 antibody. Alix was the exosome loading control. CRL-11372 was the control cell line (C) Lysed exosomes were analyzed by immunoblotting with anti-p27 antibody. Flotillin was the exosome loading control. HFL1 was the control cell line.

We further confirmed p27 in secreted exosomes using three human osteosarcoma cell lines (143B, HOS, and MG63). After isolating exosomes growth media, we conducted western blot analysis (Section 2.4) using antibodies against Alix and p27. Figure 3.3 shows the protein expression of lysed 143B, HOS, and MG63 exosomes. This immunoblot confirms osteosarcoma cells are expressing p27 (27 kDa) in secreted exosomes as well as the exosome marker Alix (100 kDa).



**Figure 3.3 p27 is expressed in exosomes released by human osteosarcoma cells.** Exosomes were isolated from 143B, HOS, MG63 human osteosarcoma cell lines via ultracentrifugation. Lysed exosomes were examined by immunoblot analysis after protein concentrations were calculated using a BCA protein assay (Section 2,5) with anti-p27 antibody and Alix as an exosome marker.

### 3.3 Exosomes are taken up by HFL1 cells

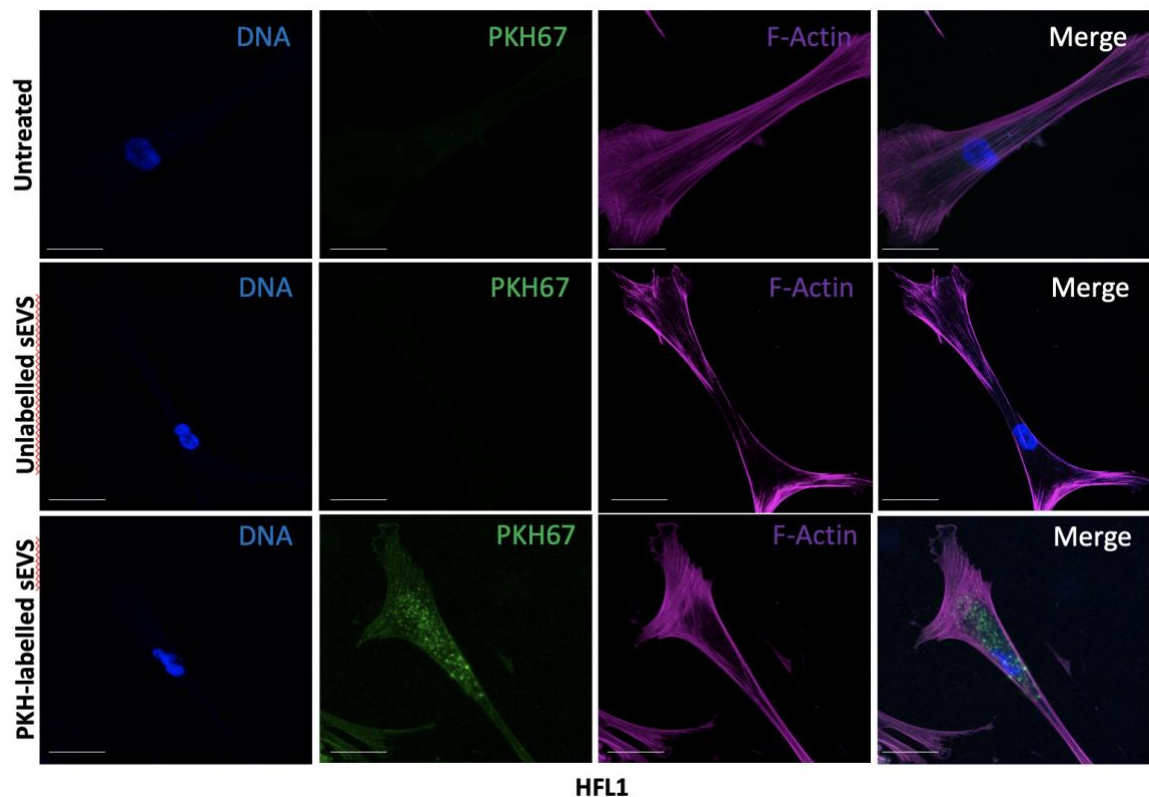
After confirmation of p27 expression in osteosarcoma derived exosomes I examined how these nanoparticles interact with cells in the lung. Since exosomes can participate in receptor ligand binding and/or be directly taken up by the recipient cell (Sinha et al., 2021). I investigated if the exosomes are taken up into recipient cells or if the exosomes remain in the extracellular matrix surrounding recipient cells. To test this, I fluorescently labeled 143B derived exosomes with PKH67 (Section 2.5) then incubated the exosomes with HFL1 cells to visualize uptake. Confocal microscopy images (Figure 3.4) show that exosomes are in fact taken up by HFL1 cells and remain contained in the cytoplasm of the cells (shown in green), suggesting that once the exosomes are taken up by recipient cells, their cargo is then released in the cytoplasm and impact the cells internally.

Once I confirmed exosome uptake in the cytoplasm of HFL1 cells, I investigated where p27 was localized in the cell following internalization. 143B derived exosomes were fluorescently labeled (Section 2.5) using an antibody against p27 and CD9. Figure 3.5 confirms the existence of exosome-associated CD9-positive structures in the cytoplasm. Following uptake by HFL1 cells, Figure 3.5 shows that p27 in exosome cargo is surrounding the nucleus and remains contained in the cytoplasm. Previous studies on p27 in osteosarcoma (Currier et al., 2019) found that in osteosarcoma cytoplasmic p27 acts as an oncoprotein. Figure 3.5 further supports this finding because p27 in exosome cargo localizes in the cytoplasm.

Following confirmation of exosome uptake and the presence of p27 in the cytoplasm I also examined how the introduction of these exosomes impacted HFL1 cell viability. To do this I conducted a cell viability assay (Section 2.8) on CRL-11372 and HFL1 cell lines after the introduction of 143B exosomes and 143B *CDKN1B*

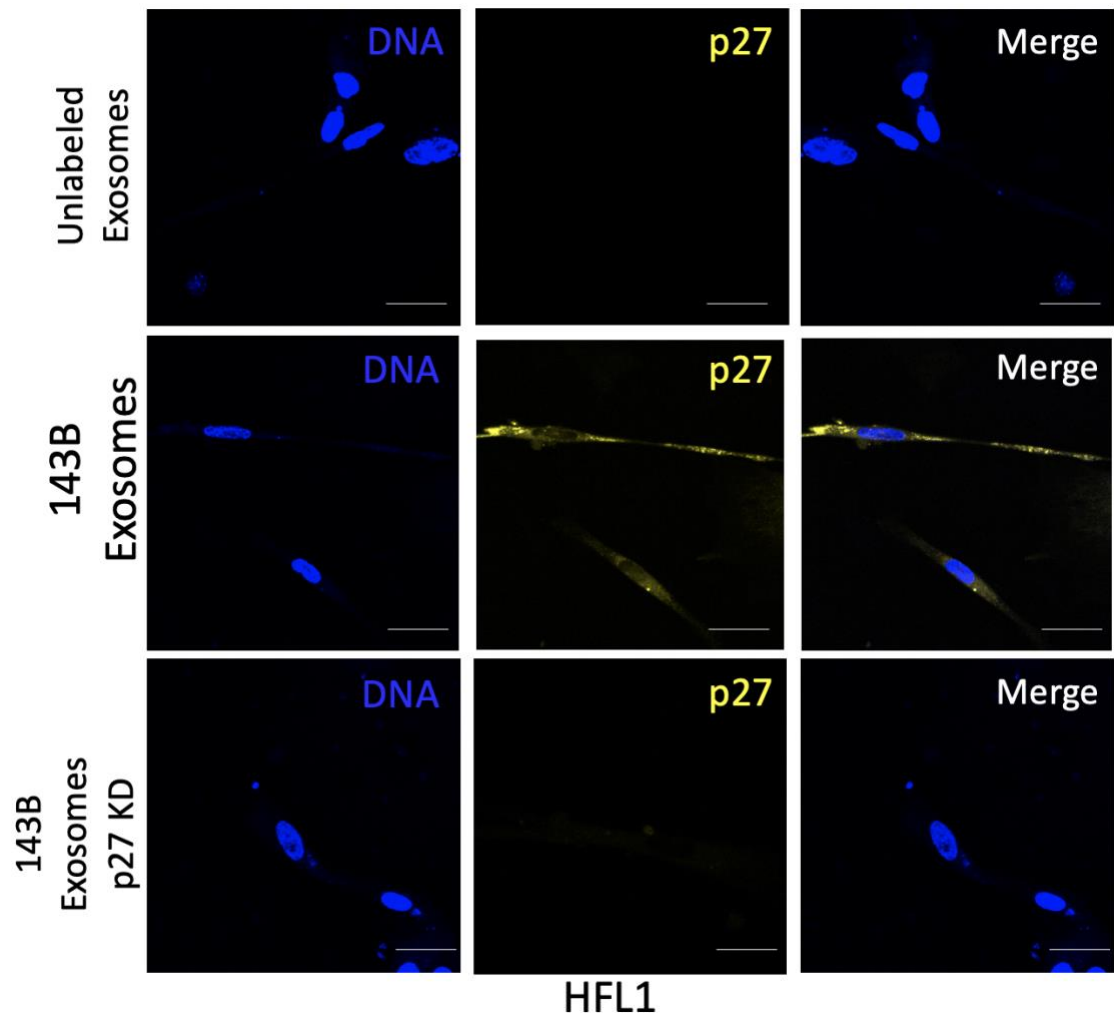
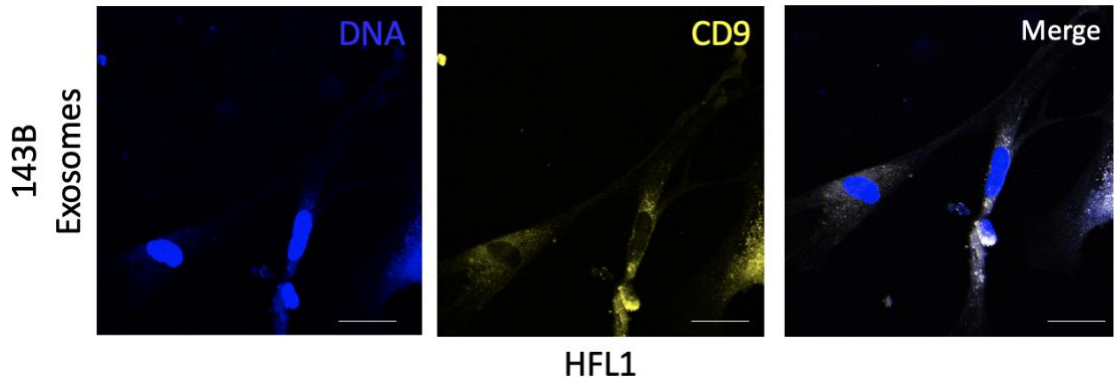
knockdown exosomes after 48 hours. The graphs in Figure 3.6, indicate that there were changes in viability of the CRL-11372 cells after the introduction of exosomes but that the viability of HFL1 cells were unaffected. While the HFL1 data does not show any significant changes, cells are viable and do not undergo apoptosis as a result of the introduction of 143B exosomes.

Looking at all of this data, it supports my second aim, that exosomes are taken up by fibroblast cells and that p27 remained localized in the cytoplasm.

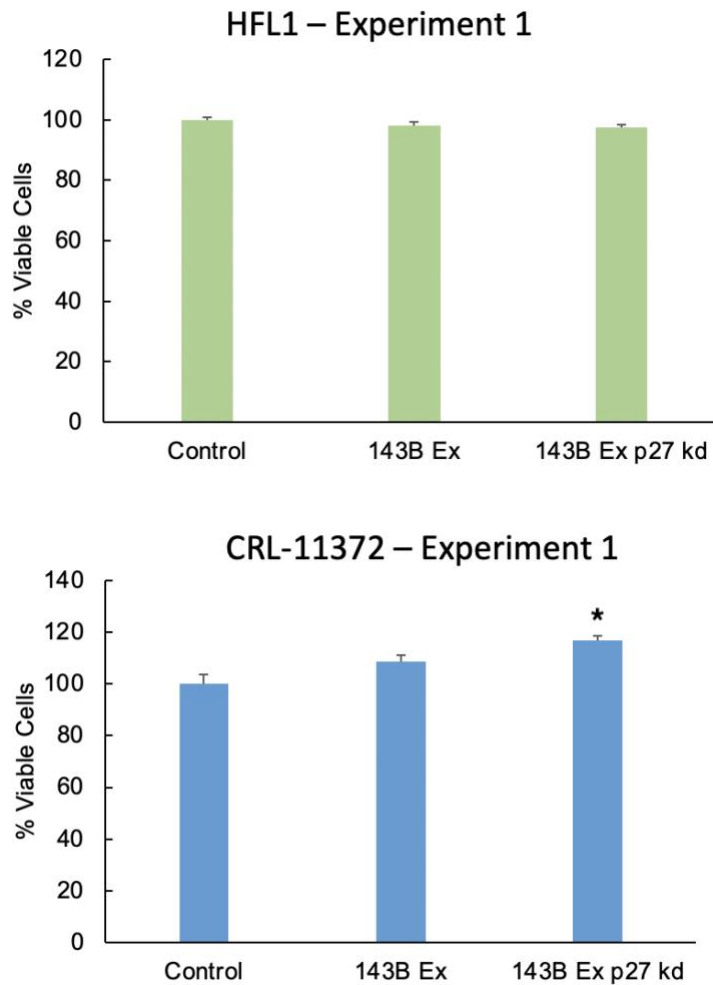


**Figure 3.4 Exosome uptake by recipient lung fibroblast cells.** (A) HFL1 lung fibroblast cells were subjected to immunofluorescence staining using PKH67 and

F-actin antibodies. Images are shown. Nuclei were stained blue using Hoechst dye.  
Magnification is 63x. Scale bars = 25  $\mu\text{m}$ .



**Figure 3.5 p27 expression is induced in lung fibroblasts after exosome uptake.** (A) HFL1 lung fibroblast cells were subjected to immunofluorescence using anti-CD9 antibody. Images are shown. CD9 is colored yellow. Nuclei were stained with Hoechst dye and are colored blue. Magnification is 63x. Scale bars = 25  $\mu$ m. (B) HFL1 lung fibroblast cells were incubated with 143B exosomes and subjected to immunofluorescence staining using anti-p27 antibody. Images are shown. p27 is colored red and nuclei are colored blue after Hoechst dye staining. Magnification is 63x. Scale bars = 25  $\mu$ m.

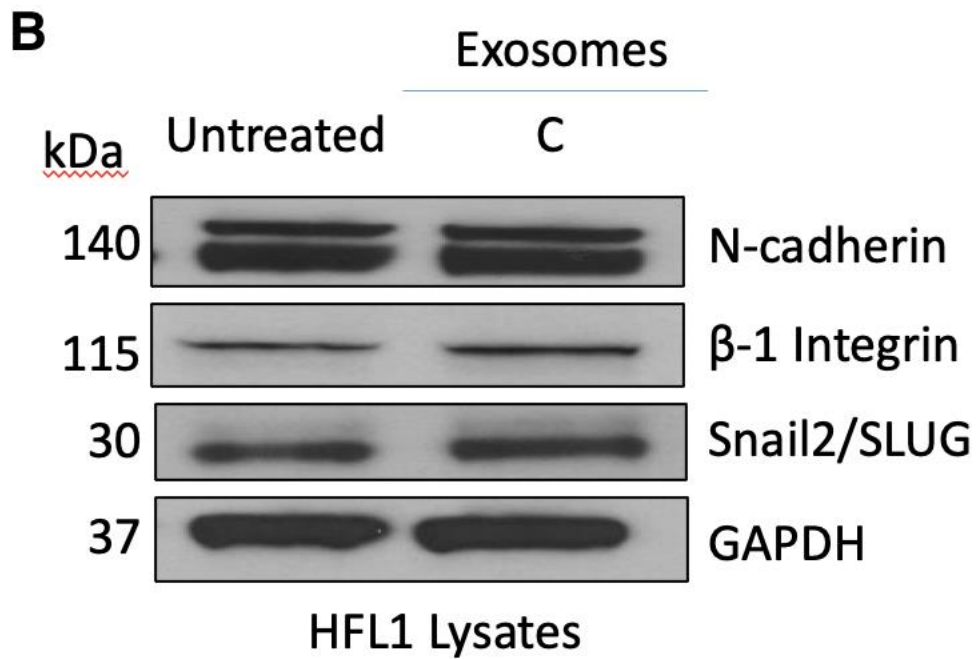
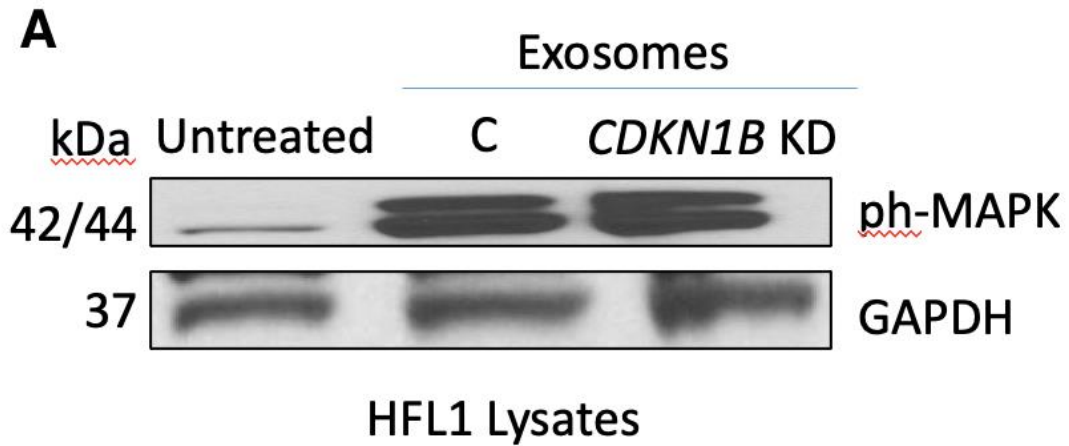


**Figure 3.6 p27 expression in osteosarcoma derived exosomes impact cell viability of lung fibroblasts after uptake.** Cell viability plots of control cell lines, lung fibroblasts (HFL1) and osteoblasts (CRL-11372) respectively after incubation with osteosarcoma derived exosomes. Control cells were incubated with osteosarcoma (143B) derived exosomes and *CDKN1B* knockdown osteosarcoma (143B) derived exosomes. Of the two experiments, the only comparison that proved to be statistically significant was between control CRL-11372 cells and CRL-11372 cells after uptake of 143B *CDKN1B* knockdown exosomes. This increase in viability could be due to other biomolecules in the exosomes influencing the recipient cells without the presence of

p27. Each bar represents the mean of 3 replicates. Error bars represent standard deviation (SD) ( $p^* < 0.05$ ).

### **3.4 Exosome uptake by human lung fibroblast cells influences cell proliferation pathways**

Now that I can confirm uptake of exosomes by HFL1 cells and that these exosomes do not induce cell death after uptake. I sought to assess how the cell is being influenced internally, more specifically the cell proliferation and the internal content. After visualization of exosome uptake (Section 3.4) by HFL1 cells I analyzed the effects the exosomes had in common cell proliferation pathways: MAPK and AKT. After exosome incubation with HFL1 cells, HFL1 cells were lysed and analyzed via Western blot analysis. Figure 3.7A shows increased MAPK signaling following exposure of HFL1 cells with 143B derived exosomes as well as p27 knockdown exosomes. This increase in MAPK signaling could be due to the exosomes having a role in promoting the epithelial-mesenchymal transition (EMT). Figure 3.7B shows gene expression for Snail2 and  $\beta$ 1-integrin was increased in HFL1 cells following exposure to exosomes, but not N-cadherin. This data suggests exosome uptake can induce distal intercellular changes in the lung environment that can alter protein expression of cells and favor tumor progression.



**Figure 3.7 Exosome uptake influences lung fibroblast content and proliferation.** (A) Lysed HFL1 cells were analyzed by immunoblotting with anti-ph-MAPK antibody. GAPDH was the endogenous loading control. (B) Lysed HFL1 cells were analyzed by immunoblotting with antibodies against N-cadherin,  $\beta$ 1-integrin, and Snail2/Slug. GAPDH was the endogenous loading control.

## Chapter 4

### DISCUSSION AND FUTURE DIRECTIONS

#### 4.1 Discussion

For a patient with early diagnosis of osteosarcoma, treatments with chemotherapy and surgery renders their chances of survival high, but when metastatic lesions develop, typically in the lung, survival drops significantly. Treatment of relapsed disease remains limited to salvage chemotherapy and further surgery to remove the lesions. This ultimately results in poor patient outcomes and in many cases patient death. Drug and therapeutic development have many hurdles. Unfortunately, the existing disease targets have not generated successes in recent clinical trials and have not proven to be sufficiently helpful for the management of disease. Further investigation is required to identify new disease targets.

In particular, nanovesicles called exosomes that are released from all cells, have shown to be a potential sources of new tumor biomarkers (Figure 4.1). Exosomes are extracellular vesicles that range in diameter of 30 – 150 nm. These extracellular vesicles are secreted by cells into the bloodstream and other body fluids and are released at a higher rate by cancer cells *in vitro* as well as in plasma of patients with tumors (Logozzi et al., 2017). These vesicles are lipid bound and contain biomolecules varying from DNA, RNA (including mRNA and miRNAs), proteins and metabolites that are reflective of the cell of origin. Exosomes are being studied as a delivery system for tumor biomarkers. The conditions of hypoxia, acidity, and low nutrient supply all contribute to microenvironmental changes that result in the increased

exosome release by tumor cells, and upregulation of known tumor markers. This is evident for prostate specific antigen (PSA) for prostate cancer (Logozzi et al., 2019), and proteins related to ion/proton transport (e.g., V-ATPase, CA-IX) (Iwasaki et al., 2015; Zhang et al., 2015). Ultimately exosomes have a potential to track cancer specific genetic information throughout the body of a patient and they can be obtained non-invasively through the use of liquid biopsies.

At present, there is no clinical data supporting the use of exosomes for the management of osteosarcoma. Existing biomarkers are not expressed with sufficient frequency to define osteosarcoma. Exosomes express specific markers of the endosomal pathway, including tetraspanins (e.g., CD63, CD9, and CD81), as well as heat shock proteins (HSP70) and proteins from the Rab GTPase family, Flotillin and Alix, which are not detectable in other types of vesicles of similar size. To identify a key role of exosomes as a source of osteosarcoma tumor biomarkers, it will be necessary to study unique exosomal proteins and nucleic acids and validate the importance of the expression of known tumor biomarkers when expressed on exosomes. Raimondi et al. have identified miRNAs packaged in osteosarcoma-derived exosomes, specifically miR-148a and miR-21-5p that affect the TME (Raimondi et al., 2020). We selected to p27 a known cell cycle inhibitor protein which is involved in the G<sub>1</sub> stage of the cell cycle and inhibits CDK complex activity causing cell cycle arrest at the G<sub>1</sub>/G<sub>0</sub> checkpoint.

Although the cause of osteosarcoma development is unknown, there is a clear relationship between the loss of osteoblast differentiation and tumor malignancy that involves p27. In eukaryotic cells, p27 acts as a cell cycle regulator that binds CDK4/cyclin D, to prevent phosphorylation of retinoblastoma protein (pRb) and

arrests cells at G<sub>1</sub>-phase (James et al., 2008). Subsequent degradation of p27 by S-phase kinase-associated protein 2 (Skp2), allows progression into mitosis. Studies by our group (Currier et al., 2019) and others (H. Zhao et al., 2014), have characterized additional roles for p27 in cell survival, differentiation, and migration. These are often described as non-canonical p27 functions since they are independent of CDK/cyclin regulation. A central feature in describing the non-canonical activity of p27 is its cytoplasmic localization. Cytoplasmic p27 participates in cellular processes including cytoskeleton dynamics, cell invasion and migration (Sharma & Pledger, 2016). High cytoplasmic p27 expression is associated with poor survival for metastatic melanoma (Denicourt et al., 2007), lung (Podmirseg et al., 2019), and prostate cancer (Ananthanarayanan et al., 2011). It appears that p27 can function as a tumor suppressor or oncoprotein that promotes development or tumorigenesis, depending on interactions in the particular cellular context. Previous research on osteosarcoma found that p27 localizes out of the nucleus into the cytoplasm thus altering p27 function from a tumor suppressor to an oncoprotein (Currier et al., 2019). Recent research has established exosomes as an alternative mediator of cell-to-cell communication within the tumor microenvironment (TME) and cancer metastasis. We investigated whether we can utilize p27 as a biomarker within exosome cargo to track osteosarcoma metastasis out of the bone to other systems in the body.

In this study, I examined the cargo of osteosarcoma derived exosomes with a specific focus on p27. I used immunoblot analysis to show that in the exosomes secreted by osteosarcoma cells p27 is expressed in the cargo, but not in those with p27 protein knockdown or those secreted by osteoblasts or normal fibroblasts.

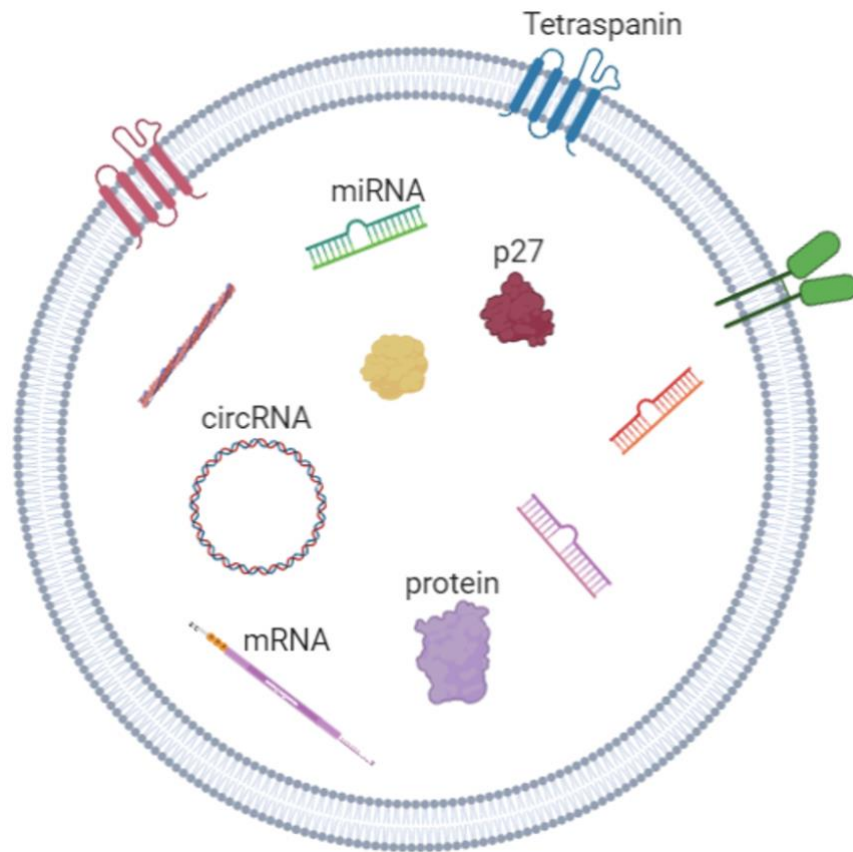
After confirmation that p27 is contained in the cargo of exosomes secreted by osteosarcoma cells, I further examined how they influence lung fibroblast cells to show the cellular changes that occur once the exosomes are in the lung. Data from confocal images taken of lung fibroblast cells, showed that exosomes were taken up directly by the cells into the cytoplasm instead of remaining in the extracellular space and that those exosomes contained p27 in their cargo. Once the exosomes are contained in the recipient cells, their cargo would be released and impact the cells internally. To analyze the impact of the introduction of these exosomes, a cell viability assay was performed to assess the direct proliferative effects in both osteoblasts and lung fibroblasts. This analysis showed that the osteoblasts had an increase in viability and the lung fibroblasts remained unchanged but there was no cell death of the lung fibroblasts. This indicates that in the lung fibroblasts, the osteosarcoma derived exosomes did not negatively impact cell survival or cause the induction of apoptosis.

Our study has also shown an increase of protein levels of metastatic markers, specifically Snail2 and  $\beta$ -1 integrin, but not N-cadherin in the cell lysates of HFL1 cells using western blot analysis. Intercellular communication is a key feature of tumor progression and metastasis. Tumor derived exosomes express multiple proteins, including oncogenic proteins, integrins, and signaling molecules (Wortzel et al., 2019). Analysis of exosomes from osteosarcoma cells also revealed the exosomal packaging of proteins associated with cell proliferation, invasion and metastasis (data not shown). Collectively, these studies demonstrate how transfer of tumor derived exosomes can elicit alteration that are associated with MAPK signaling to promote changes in the lung fibroblasts for osteosarcoma. These can potentially lead to increased motility, invasiveness, and stemness of cells targeted by these exosomes to

promote the formation of tumor niches in target organs, to induce malignant transformation and cancer progression. Taken together, these data show that exosomes released from osteosarcoma cells have a prominent role in transferring cargo that favor both the growth of primary tumors and their metastatic spread. They provide a source of biomarkers and represent targets for future treatment strategies.

#### **4.2 Future directions**

Our research supports the study of exosomes as liquid biopsies for osteosarcoma. Further targets can be used to develop techniques to quantify and characterize plasma exosomes using special techniques to purify osteosarcoma specific exosomes. Careful statistical analysis studies can be performed to compare exosome p27 levels from primary and metastatic tumors, to plasma exosomes to show that the exosome-related measures are significantly correlated and that exosome p27 values reflect the same biological status of tumors. These data highly support the use of current technologies for both characterization and quantification of circulating exosomes, providing potential new sources of clinical biomarkers for screening tests, diagnosis, and follow-up of patients with osteosarcoma, and possibly also patients with other diseases.



**Figure 4.1 Overview of exosome composition and cargo.** Exosomes are small extracellular vesicles with a diameter range of 30 – 150 nm and have a lipid bound membrane. This membrane has specialized receptors and specific membrane associated proteins including tetraspanins. These small vesicles have cargo that contain a multitude of biomolecules, DNA, RNA, mRNA, miRNA, and various proteins. Figure is generated with BioRender.

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