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Exosomes And Their Role In Asbestos Exposure And Mesothelioma

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EXOSOMES AND THEIR ROLE IN ASBESTOS EXPOSURE AND MESOTHELIOMA

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ABSTRACT

Malignant mesothelioma (MM) is a locally invasive and highly aggressive cancer arising on the mesothelial surface of organ cavities (mainly pleural) as a direct result of asbestos exposure. The latency period of MM is long (20-50yrs) after initial asbestos exposure, and the prognostic outcomes are dismal with median life expectancy of 6-12 months post-diagnosis. There are no useful biomarkers for early MM diagnosis, no successful therapeutic interventions. These vast voids of knowledge led to our hypotheses that secreted vesicles, termed exosomes, play an important role in MM development and tumorigenic properties. Exosomes are nano-sized particles secreted from all cell types and carry biologically active cargo in the form of proteins, RNA, and lipids that can potently act as intercellular messengers in both healthy settings and disease states. We are the first to have conducted studies implicating the roles of exosomes in MM pathogenesis.

Firstly, we analyzed the proteomic signature of exosomes from asbestos exposure models, in vitro and in vivo. Our in vitro data demonstrated that asbestos exposed lung epithelial cells and macrophages secrete exosomes with differentially abundant proteins compared to non-exposed controls and some of these proteins are relevant to asbestos exposure toxicology and MM development. Additionally, the exosomes from asbestos exposed cells significantly modulated the gene expression of target mesothelial cells in a way that reflected epithelial to mesenchymal transition and other tumorigenic properties. The in vivo mouse studies illustrated that mouse serum exosomes house differentially abundant proteins after asbestos exposure and this is measurable at an organism wide scale.

Secondly, we assayed the miRNA composition of MM tumor exosomes compared to healthy mesothelial cell exosomes and found signature differences in miRNA abundances, particularly that MM tumor cells had significantly higher amounts of tumor suppressor miRNA, particularly miR-16-5p, in their exosomes. This led to the hypothesis that MM tumor cells preferentially secrete tumor suppressor miRNAs via exosomes to rid themselves of the anti-tumor effects. We employed exosomes secretion inhibitors and exosome force-feeding to demonstrate that MM cells do in fact secrete miR-16-5p (along with other tumor suppressor miRNAs) through exosomes and that this property can be targeted as a potentially novel therapeutic advance. Furthermore, we identified a mechanism of miR-16-5p loading into exosomes by the RNA binding protein HuR, and this mechanism is interestingly regulated by miR-16-5p itself in a negative feedback loop.

Our studies thus far provide intriguing evidence on the role of exosomes in asbestos exposure and MM biology. We demonstrated the potential for exosomes as protein biomarkers in asbestos exposure and conduits of tumorigenic information to mesothelial cells. In addition, we incriminate exosomes as vehicles of tumor suppressor removal from MM tumor cells and we can target this as a potential n MM therapy.

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Asbestos

Any report worth its salt on asbestos induced disease including malignant mesothelioma (MM) must begin with a clear description of asbestos fibers themselves. Asbestos fibers are naturally occurring silicate particles named from the Greek “inextinguishable”, due their flame retardant properties and supreme insulator capacity. Asbestos has been documented as being used in over 3,000 manufacturing purposes; mainly in construction. The versatility of this fiber comes from its resistance to destruction, decomposition, and overall tensile strength (1). As will be discussed shortly, the wide-ranging use of asbestos was a major folly of man that has had broadly detrimental impacts on human health.

There are two groups of the hydrated silicate fibers referred to as asbestos, serpentine and amphibole, both of which are delineated as having length to width ratios greater than 3:1. Chrysotile asbestos belongs to the serpentine group (referred to as such due to their curly fibers) and is the most widely used type of asbestos, accounting for nearly 95% of all mined and manufactured. The amphiboles are straight fibers, and among them are crocidolite and amosite. Although all types of asbestos are dangerous, crocidolite is accepted as being the most carcinogenic. Moreover, not only are there structural dissimilarities between the two classifications of asbestos, there are chemical differences too. Chrysotile (serpentine) asbestos’s chemical formula is $Mg_3Si_2O_5(OH)_4$ and crocidolite is $Fe_2H_{16}Mg_3Na_2O_{24}Si_8+$.

By 1960, it was discovered that asbestos was toxic (2) and eventually was classified as a category 1 carcinogen by International Agency for Research on Cancer (IARC). The toxicity of asbestos fibers is directly associated to two main factors: the geometry and

chemical makeup. As the direct route of asbestos exposure is inhalation, fibers become deposited in the upper respiratory tract where they interact with epithelial cells and lung resident macrophages (3, 4). The fibers' length and width aspect ratios determine how the cells interact with the fibers, whether by attempted phagocytosis or if the fiber causes inflammation through physical contact with the cell surface leading to ensuing inflammatory cascades (4, 5). It is understood that longer, thinner fibers migrate deeper into the lung, whereas shorter fibers remain lodged in the upper respiratory tract. Furthermore, the resultant toxicity of the fibers is greatly dependent on the production of reactive oxygen species in the lung, as caused by the redox potential of the fibers. Free radical production is a hallmark of asbestos exposure in the lung and, although all asbestos classification cause this, crocidolite is the most dangerous, perhaps due to the prevalence of iron, which based on Fenton reactivity will lead to significant radical production (6-8).

Multiple diseases are the direct result of inhaled asbestos fibers. Pulmonary fibrosis, or asbestosis, begins with initiation at contact sites of alveolar epithelial cells, has a latency period of 20-30yrs, and presents itself most commonly at the sub pleural level in the bronchioles. A tenant of asbestos exposure, commonly observed in asbestosis patients' thoracic lymph nodes, is the presence of golden-brown rode-shaped beads referred to as asbestos bodies. The development of asbestosis causes chest-tightness and pain as the lung tissue becomes fibrotic and hardened, and leads to restricted pulmonary function. Asbestosis cases in the USA are increasing although asbestos use is overall declining, and asbestosis remains in possible association with future development of lung cancer or mesothelioma (9).

Other pleural diseases caused by asbestos include, pleural effusions and pleural plaques/lesions. A small number of asbestos exposed individuals develop pleural effusions, and this occurs up to 20 years after high concentrations of exposure. Pleural plaques are rather common developments of asbestos exposure and are composed of hyalinized fibrotic tissue. These plaques are common in the intercostal space of the thorax and diaphragm. These are a result of inflammation of the pleura and have a latency of about 10 years after exposure (10, 11).

Additionally, lung cancer is associated with asbestos exposure. The cases of lung cancer associated with asbestos exposure appears to be directly correlated with smoking cigarettes. The combined effects of asbestos and smoking appear to be super-additive or even multiplicative, due to the 90-fold increase of predispositions of lung cancer in smokers and asbestos exposed individuals (12-14).

The most aggressive disease caused by asbestos exposure, however, is the cancer referred to as malignant mesothelioma.

1.2 Malignant mesothelioma

Malignant mesothelioma (MM) is a highly aggressive tumor that originates on the mesothelial surface of organ cavities, and is the causative result of asbestos exposure in the majority of cases (15). MM predominantly arises in the thoracic cavity (85% of cases), or pleural surface, hence named malignant pleural mesothelioma (MPM). The other, less common tissue origins of MM are on the peritoneal cavity, pericardial surface, or tunica vaginalis (16). Due to its prevalence and the focus of the subsequent experiments

performed in the following thesis work, the focus will pertain primarily to MPM. An interesting note is that. MPM develops on the parietal surface of the pleural mesothelium, not the visceral surface.

The latency period for MM development is between 20-50yrs after initial asbestos exposure, which correlates with the other asbestos related diseases as not immediately arising. The incidence of MM is on the increase worldwide, although reports by governmental agencies had initially predicted a plateau in 2015 followed by steady decreases. This lack of clairvoyance is likely due to the fact that asbestos is so prevalently common in nearly all geographic settings internationally, regardless of declining rates of asbestos mining of new manufacturing processes. Additionally, the unregulated asbestos use in industrial countries such as China, Russia, and India will add to the global upward trends in MM to a significant degree.

Early diagnosis of MM is nearly impossible due to lack of biomarkers, and prognostic outcomes are grim with median survival post-diagnosis being around 12 months because of no successful therapeutic interventions. The large proportion of MM patients are male (75% or more) with median age of 73 (17). This statistic is due to the fact that asbestos exposure traditionally occurs in occupational settings such and factories, manufacturing plants, and in the military that are predominantly male dominated.

MPM patients are commonly diagnosed after symptoms have developed such as breathlessness, painful breathing, chest-wall pain, and there is a typical presentation of pleural effusion or ascites (18). Unfortunately for patients, diagnosis after symptom onset

can take time prior to cytological analysis or is incidentally discovered via chest radiography.

Along with asbestos exposure, there are implications that simian virus 40 (SV40) is involved in MM development via the blockage of tumor suppressor genes, making SV40 a rather potent oncogenic virus linked to MM (19). In addition, there have been rare instances where radiation led to MM.

It is estimated that there are 2 billion mesothelial cells in the human body, the origin sites for MM development. The function of healthy mesothelial cells is to allow friction free movement of organ tissues when moving against one another. Asbestos fibers, as mentioned, contact to lung epithelial cells and resident macrophages initially, so there are a few ideas as to how the assault of asbestos in the lung leads to tumorigenesis at a site on the surface of the lung. Once in the lung, it is suggested that they can be dragged outward towards the pleura via pulmonary lymph flow and become translocated near to or on the pleural surface (20).

There are four main suggestions as to how asbestos damages the pleura, leading to tumorigenic changes (prior to the subsequent evidence defined within this thesis). First is that based on the geometry of asbestos fibers, they penetrate and damage/irritate the pleura. Second, by genotoxic effects and mitotic disruption by the fibers interacting with mitotic spindles, perhaps a result of partially phagocytosed fibers and direct piecing of the cell, causing chromosome damage. Third, ROS-based DNA damage from fibers that signals to and disrupts the redox state of mesothelial cells. And fourth, signaling via MAP kinases and extracellular signal–regulated kinases (ERK) (21-23) or other signaling molecule(s).

The uncontrolled growth of MM is attributed to multiple factors such as telomerase expression in majority of mesotheliomas, self-production of growth factors (platelet derived growth factors, epidermal growth factors, and Wnt pathway proteins) (24), and a very prevalent tenant of MM is the loss of tumor suppressor genes (25).

The fact that only 5% of asbestos exposed individuals develop MM, insists that genetic predisposition also plays a role. One of the most well-delineated mutations indicating MM susceptibility is BRCA-associated protein 1 (*BAP1*). Families with BAP1 mutations have strong dispositions for MM development, among other cancers, meaning that BAP1 germ line mutations are also a form of hereditable multi-cancer syndromes. There are also other lower risk susceptibility genes for MM such as XRCC3, NAT2, and GSTM1. Additionally, tumor-suppressor gene mutations in LATS2, NF2, and CDKN2 are potent somatic mutations identified in patients who went on to develop MM (16, 25-27).

Diagnosing MM is a challenging topic as it can be commonly mischaracterized as a carcinoma and because there are no useful means of early detection (i.e. biomarkers for early development or asbestos exposure). One of the first steps in diagnosis involves radiological imaging for localization and staging information. Cytological analysis is a crucial step, although not perfect, to assess the immunohistochemical makeup of a tumor ascitic fluid sample. The sample is assayed for the presence of mesothelial markers such as Wilms' tumor antigen and further for markers of malignancy such as epithelial membrane antigen (EMA) (28). The inconclusive nature of such sampling typically necessitates biopsy and histopathological analysis. Staining of samples for cytokeratin aids

in ruling out sarcoma or melanoma, and further characterized by specific antibodies to MM such as EMA, calretinin, and mesothelin (a highly common MM marker) (29).

The current state of biomarkers for MM is dismal, as none yet are definitively useful for early diagnosis or accurate prognostic indications. The cell adhesion glycoprotein, mesothelin, which is overexpressed in MM cells has a soluble form that can be elevated in the serum of MM patients. However, mesothelin is only seen to be of use in advanced stages of epithelioid MM, and not in other subtypes (30). Another potential biomarker in MM is osteopontin, which is involved in cell-matrix interactions and has been shown to be elevated in serum of MM patients, but is not a clear distinguisher between other pleural diseases (31). Fibulin-3, another possible biomarker, was also shown to be highly increased in MM patient plasma, but not as accurately as mesothelin (32, 33). Although these serum markers have showed some promise, they are not strong enough indicators of disease to be deemed bona fide biomarkers. The field of MM and asbestos exposure is in need of an accurate biomarker to make early diagnosis; any such evidence would be a crucial advance.

The diagnostic and prognostic outcomes of patients is also related to the subtype of cancer and MM is capable of exhibiting three separately identifiable subtypes: epithelioid, sarcomatoid, and biphasic or mixed. Epithelioid MM cells are polygon, cuboidal, or oval and can appear similarly to non-cancerous mesothelial cells in some cases. The most common form of MM is epithelioid (80% or more of cases) and is the least aggressive with best prognostic outcomes.

Sarcomatoid cells are more spindly in morphology and can appear to show elements of sarcoma cells. Sarcomatoid MMs are the least common and least responsive to

chemotherapeutic intervention and are more aggressive in that they grow faster and have a higher invasive capacity. Biphasic MM consists of a mix of both types within the same tumor, and patient life expectancy is higher when a larger portion of the tumor tissue is composed of epithelioid cells (29).

There is currently no curative option in MM, and the standard of care is chemotherapy, radiotherapy, and surgery. Chemotherapy is the treatment option most commonly employed and has the most evidence of increasing MM patient survival. The common regimen of chemotherapy in MM utilizes cisplatin or pemetrexed, or a combination of the two. The combination of the chemotherapeutic drugs showed outcomes of up to month longer survival rates, and cisplatin alone showed a respective increase of about 9 months (34). Subsequent clinical trials with other drugs such as raltitrexed with cisplatin showed similar outcomes, but no change in chemotherapeutic standard of care has emerged from these trials (35).

MM has been shown to be relatively resistant to most radiotherapy treatments, but locally directed radiation can be helpful as palliative care in some cases. As an adjuvant to chemotherapy or surgery, the results are mixed and there is a high risk of toxicity, so this mode of treatment is limited and unlikely to expand or continue (36).

There exists disparate points of view on the usefulness of surgery in MM. In some cases, surgery is helpful for palliative care, and others state that surgery is only helpful in the setting of combined therapy such as with chemotherapeutics or immunotherapy. The two approaches to surgery in MM are removal of visible disease and debulking procedures to spare nonmalignant tissue, or a more extreme approach of extrapleural pneumonectomy

(EPP) to remove any and all traces of potentially malignant tissue. EPP has shown some promise for enhanced survival, but complication rates and mortality from surgery are high leading it be a rare option (37, 38).

There is increasing ardor in the field of MM, as with all cancers, to treat the disease with immunotherapy. One of the most promising areas of such treatment lies in checkpoint blockade inhibitors, because tumor cells commonly upregulate surface receptor expression of inhibitory ligands that prevent immune cells from targeting them. This adaptive advantage of tumor cells is a popular site amongst researchers to target with antibodies that prevent the inhibitory signaling. Such checkpoint blockade inhibition would thusly prevent immune cell inhibition and allow the immune system to target the cancer. In the context of MM, cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein-1 (PD-1)/programmed cell death protein ligand-1 (PD-L1) have been investigated as possible therapeutics (39).

Another approach of immunotherapy is to attack tumor cells with antibodies fused to potent toxins, termed immunotoxins. Because mesothelin is overexpressed in MM, mesothelin-specific antibodies have been linked to toxin SS1P and has shown some anti-tumorigenic effects, especially in combination with chemotherapy (40, 41). Additionally, some oncolytic viral therapies have been suggested in MM, as have anti-cancer vaccines via the exposure of dendritic cells to tumor antigens to produce immune response (42).

One of the most exciting advances in cancer immunotherapy involves adoptive cell therapies, where patients' own immune cells are isolated and engineered ex vivo to expand into cells that can target tumor cells. These modified immune cells are reintroduced to the

patient as a means of treatment. Such immunotherapies harness T cells either by genetically modulating T-cell receptors (TCR) or chimeric antigen receptor (CAR) modified T cells (43). Currently there is one clinical trial for TCR targeting WT1 in MM; there are only limited antigens to target using TCR so the applicability is minimal. The limitations of TCR are non-existent in CAR T cell based approaches because T cells can theoretically be modified to target any antigen via this approach. There is a current trial testing CAR T cells against the antigen mesothelin which is overexpressed on the surface of many MM tumor cells. Mesothelin-targeted CAR T cell therapy in this sense harnesses the potential of T cells to attack tumors with this antigen, and the delivery method is an intrapleural single dose. Intrapleural delivery was shown to be more effective in mouse models studies leading up to the clinical phases as compared to systemic delivery; regional delivery eradicated tumors with 30-fold lower dose (44).

Another important facet of this thesis work revolves around microRNA (miRNA), small non-coding RNA molecules about 20-25 nucleotides in length that are vital regulators of gene expression at the post-transcriptional level. The function of miRNAs is that they bind to mRNA molecules and either prevent their translation at the ribosome, or direct the mRNA to be degraded. The specificity of miRNAs is based on its complementary base pairing with mRNA, and because there does not need to be 100% fidelity of complementarity, each miRNA will have multiple targets, and every mRNA may be targeted by multiple miRNAs. In this gene regulatory sense, miRNAs can act as potent oncogenes or tumor suppressors depending in their targets. The levels of miRNA in tissues and cells, along with those that are secreted, can therefore be of important diagnostic and

prognostic value. The underlying biology of many tumors, including MM, is a result of dysregulated miRNA expression, and understanding fully the scope of miRNAs in these disease settings can be crucial for making therapeutic advances and identifying drug targets or biomarkers.

Most commonly, tumor suppressor miRNAs are under-expressed in MM tumor cells and tissues. The targets of such miRNAs in MM are oncogenes involved in tumor progression and development, such as CCND1, BCL2, CDKN2A, NF2, JUN, HGF, and PDGFA, and these results have been shown across various studies in the previous decades (45). There are a number of well-defined miRNAs whose expression is nearly lost in MM including, miR-15, miR-16, miR-203, let-7, miR-31,, miR-126, miR-135b, miR-181a-2, miR-499, miR-517b, miR-519d, miR-615-5p, and miR-624. Within this listed set, and others not mentioned here, is a series of targeted pathways that are vital to tumor growth and progression. For examples, miR-126 targets VEGF mRNA and thereby prevents angiogenesis, but MM patients, who tend to show increased levels of VEGF in serum, have downregulated miR-126 in their tumors. This type of miRNA-based deregulation is important to MM biology, as it appears to be another type of genetic or mechanistic evolutionary growth advantage. By having low, or non-existent, levels of tumor suppressor miRNAs, MM tumor cells are more capable of uncontrolled growth and proliferation.

miRNAs also play an important role in profiling MM tumors and in potential biomarker identification. It has been shown that certain miRNAs are useful for differentiating MM from carcinomas (miR-200, -141, and -429), and in identifying various MM subtypes (46). For this matter, MM miRNAs could be helpful as diagnostic tools. Circulating miRNAs

are also becoming a more promising route for early detection, and in MM it has been seen that certain miRNAs (miR-103 and miR-625) are capable of distinguishing MM patients from asbestos exposed or healthy controls and differentiating MM from fibrosis (47).

One well-defined miRNA aberration in MM is the loss of miR-16 expression. This miRNA is a potent tumor suppressor that functions by targeting the expression of genes such as CCND1 and BCL2. Multiple studies have shown that miR-16 is significantly under-expressed in MM tumors and cells as compared to healthy controls. More intriguingly still, miR-16 is becoming a focus of potential miRNA based treatment strategies by reintroducing the miRNA back to tumors to induce the pro-apoptotic function (48). This is an exciting strategy that employs the fact that a targeting therapeutic strategy is possible by using the tumor suppressor miRNAs that MM has lost.

The dysregulated landscape of miRNAs in MM is of much interest in the biomarker and treatment fields of MM and much is yet to be explored. The thesis work herein will shed some light on a subset of miRNAs in MM within the context of those that are secreted in vesicles called exosomes.

1.3 Exosomes

Exosomes: Potential in Cancer Diagnosis and Therapy

Exosomes: Potential in Cancer Diagnosis and Therapy. Medicines. 2015 Dec; 2 (4): 310-327. PMID: 27088079 Exosomes in Cancer and Therapeutic Potentials

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Abstract

Exosomes are membrane-bound, intercellular communication shuttles that are defined by their endocytic origin and size range of 30-140nm. Secreted by nearly all mammalian cell types and present in myriad bodily fluids, exosomes confer messages between cells, proximal and distal, by transporting biofunctional cargo in the form of proteins, nucleic acids, and lipids, and play a vital role in cellular signaling in both normal physiology and disease states, particularly cancer. Exosomes are powerful progenitors in altering target cell phenotypes, particularly in tumorigenesis and cancer progression, with the ability to alter tumor microenvironments and to establish the pre-metastatic niche. Many aspects of exosomes present them as novel means to identify cancer biomarkers for early detection and therapeutic targets, and using intrinsic and engineered characteristics of exosomes as therapeutic devices to ameliorate the progression of the disease. This review outlines some of the recent and major findings with regards to exosomes in cancer, and their utilization as therapeutic opportunities.

Keywords: Exosomes; cancer; therapy; biomarkers; tumor microenvironment; pre-metastatic niche keyword; keyword (3-10 keywords separated by semi colons)

Introduction

The ability for cells to communicate is quintessential to the biology of multicellular organisms. Intercellular signaling events are accomplished in many ways and depend on a complex array of networks and processes including direct contact, electrical and chemical components, soluble molecular messengers, or the secretion of membrane-bound vesicles. An emerging field in biological research focuses on exosomes as a seminal conduit for this cellular crosstalk. Exosomes were first described in 1981 as "exfoliations" from neoplastic cell culture monolayers (49), and have since gained significant momentum for their biological and therapeutic relevance. Nearly all mammalian cell types have been shown to produce exosomes and their presence has been confirmed in many bodily fluids, including urine, blood, saliva, and amniotic fluid (50).

Exosomes are small, 30-140nm, membrane bound particles defined by their origin from the endosomal pathway (Figure 1), and are not to be confused with microvesicles which are larger (~1,000nm) and are shed directly from the plasma membrane (reviewed in (51)). The content of exosomes is another pivotal feature of their classification and ability to carry information and cargo, as they are enriched in RNA species (i.e. mRNA, miRNA), proteins, biofunctional lipids, and occasionally DNA (52). With communication at the forefront of their function, exosomes can also participate in waste removal, antigen presentation, and the induction of pro-inflammatory cytokine release (53).

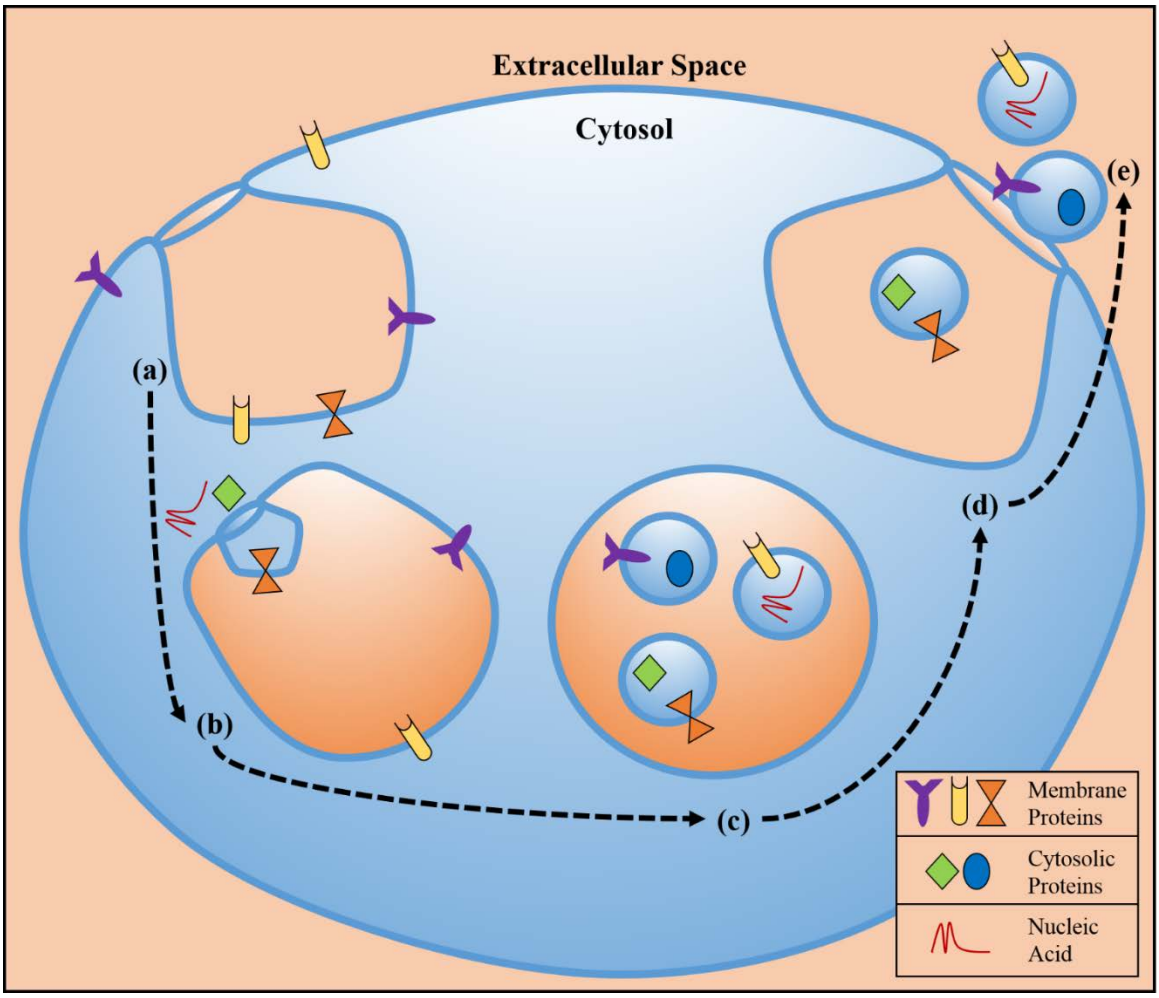

Figure 1. Schematic representation of exosome biogenesis and release, with depictions of membrane proteins, cytosolic proteins, and nucleic acid. (**a**) Endocytosis at the plasma membrane leads to the (**b**) immature endosome where invagination of the endosomal membrane occurs and molecular cargo is loaded into the newly forming particles. (**c**) The mature endosome, or multivesicular body (MVB) contains the exosomes and (**d**) upon fusion of the endosomal membrane with the plasma membrane, (**e**) the exosomes are released to the extracellular environment, maintaining the producer cell

membrane topology and housing protein/nucleic acid cargo, which then can travel to recipient cells, locally or distally, communicate molecular information and/or induce phenotypic changes.

Interestingly, exosomes are attributed to playing roles in both normal physiological conditions (immune surveillance, neural plasticity, tissue repair, stem cell maintenance, and blood coagulation pathways) as well as in the pathological processes of many disease states (54). For this review, we will focus on the role of exosomes in cancer, although they are associated with the pathogenesis of viruses like HIV-1, the progression of Alzheimer's and Parkinson's disease, the spread of prion proteins, and inflammatory conditions (55). The roles of exosomes in disease demonstrate their prospective utilization as either therapeutic targets, or potentially as therapeutic agents.

Production of exosomes occurs at the early endosome, resulting in the formation of a multivesicular endosome (MVE), however, the exact mechanisms of their biogenesis is not well understood. The early endosome is the direct product of a primary endocytic event at the plasma membrane. Invagination of the endosomal surface and subsequent pinching off of the membrane creates the exosomes, an endosomal-endosome, of sorts also referred to at this stage as intra-luminary vesicles.

Two major pathways are suggested in the production of exosomes at the endosomal membrane: the Endosomal Sorting Complex Required for Transport (ESCRT)-dependent pathway and the ESCRT-independent pathway. The ESCRT-dependent pathway requires an accessory protein ALIX and is comprised of four complexes: ESCRT-0 which identifies and loads ubiquinated proteins on the endosomal surface; ESCRT-I and ESCRT-II which

cause membrane budding; and ESCRT-III which is involved in membrane separation. The ESCRT-independent pathway is proposed to involve lipids such as sphingosine-1-phosphate and ceramide, microdomains enriched with tetraspanins, and the enzyme sphingomyelinase (56). The study by Columbo et al. went on to illustrate that disrupting certain parts of the ESCRT machinery resulted in decreased production of exosomes from cells in culture. Recent evidence reports an exosomal production pathway requiring the membrane protein syndecan and cytosolic protein syntenin, in which these two proteins interact with the ESCRT-accessory component ALIX, the GTPase ADP Ribosylation Factor 6 (ARF6), proteolipid protein D2, and the endoglycosidase heparinase (57). In conjunction with these pathways, it should be noted that there are four major requirements for exosome biogenesis: cytoskeletal components such as actin and microtubules; molecular motors such as kinesin and myosin; molecular switches which are primarily small GTPases; fusion machinery and tethering factors such as SNAREs (58).

The exosomal membrane reflects aspects of the endosomal membrane composition, membrane constituents of the parent cell, and maintains the same membrane topology as the plasma membrane. The exosomal membrane, therefore, is enriched in MVE-related proteins such as flotillins, Annexins, GTPases, Rab, and SNAREs; proteins involved in MVE biogenesis such as ALIX, Tsg101; and membrane-microdomain associated proteins, particularly certain tetraspanins (CD9, CD63, CD81, and CD82) (51). The lipid composition of exosomes is enriched in sphingomyelin, cholesterol, and ceramide. Moreover, the membrane of exosomes can also present Major Histocompatibility Complex (MHC I/II) molecules and/or antigens, depending on the cell type from which the exosome

was secreted. These specific proteins and lipid molecules are important tools in the classification of exosomes and are attractive targets for the identification of novel biomarkers (59).

The internal cargo of exosomes is noticeably dissimilar to that of the producer cell's cytoplasmic content, indicating that cargo loading into exosomes is not a simple, diffusive, or unregulated process. This selective packaging of certain proteins and RNA species into exosomes adds another layer of complexity to understanding their biogenesis and indicates a sophisticated sorting process. Only some elucidations have been made as to the relationship between certain biogenesis/sorting molecules and their respective cargo such as ESCRT-0 loading ubiquinated proteins. ESCRT-II has been shown to specifically bind mRNAs suggesting its role in the cargo sorting of mRNA into exosomes (60).

Nevertheless, analysis of proteins and RNA identified in exosomes is readily available in an online database by ExoCarta. The most commonly identified exosomal proteins are heat shock protein (HSP)-8 and CD63. Cytoskeletal proteins are commonly identified (β-actin, cofillin, moesin, and tubulins) in exosomes, as well as proteins involved in cellular signaling pathways (β-catenin, WNT5B, and Notch ligand Delta-like 4) (61). Due to the fact that cargo recruitment is not well understood, it can only be postulated that specific chaperone proteins found in exosomes, like HSC, HSP90, 14—3-3, and PKM2, are regulators of the process (62) and that other proteins are incorporated based on their interactions with lipid-raft associated molecules which become incorporated into the MVE (63).

Notably, one of the more interesting components of exosome cargo is their enriched population of small non-coding RNAs, specifically microRNA (miRNA), but others are also incorporated (piRNA, snoRNA, scaRNA, Y RNA, siRNA, tRNA fragments, vault RNA) (64). Nearly half of the genes in our cells are regulated by miRNA (65) further substantiating the signaling capacity and modulatory capabilities of exosomes on target cells.

Exosomes are released to the extracellular space upon fusion of the MVE with the plasma membrane. This process is mediated by a subset small, vesicular transport regulation GTPases known as Rab27A, Rab11, and Rab31 (66), and another reported mechanism for secretion, specifically for exosomes bearing WNT, involves the SNARE protein YKT6 (67). Alternatively, some exosomes are not released, and are instead destined for lysosomal degradation, which has been attributed to MVE lipid composition where it appears that MVEs with cholesterol poor membranes, and/or have lysobisphosphatidic acid present are targeted for the lysosome (68).

Target cell specificity is not yet fully understood but is likely determined by adhesion associated molecules present on the exosomal surface such as integrins and SNAREs, with the possible influence of tetraspanins complexes (69). There are several fates of exosomes once bound to a recipient cell, prompting what signaling information is delivered: the exosome can bind and associate with a membrane receptor or dissociate; direct fusion with the plasma membrane and unloading of cargo to the target cytosol; or endocytic internalization (51).

Exosomes and Cancer

There is substantial and mounting evidence on the dynamic role of exosomes secreted by cancer cells in contributing to tumorigenesis, disease progression, metastasis, angiogenesis, extracellular matrix (ECM) remodeling, immune evasion, chemoresistance, and the establishment of the pre-metastatic niche (reviewed in (55)) (Figure 2). Exosome secretion by tumor cells is markedly upregulated as is observed by increased exosome collection from cancer cell cultures or serum of cancer patients compared to non-cancerous conditions (70) (71). Furthermore, the epigenetic cargo of tumor exosomes is remarkably different than the exosomes secreted by the same cell types before malignancy. Tumor derived exosomes are capable of exchanging information between neighboring cancer cells and, more notably, can communicate with distant sites and various cell types. The capability of tumor exosomes to house tumorigenic information and induce distal or local cellular responses that promote disease pathogenesis make tumor exosomes an attractive tool in identifying cancer biomarkers, uncovering molecular mechanisms to cancer biology, and exploiting exosomes for therapy.

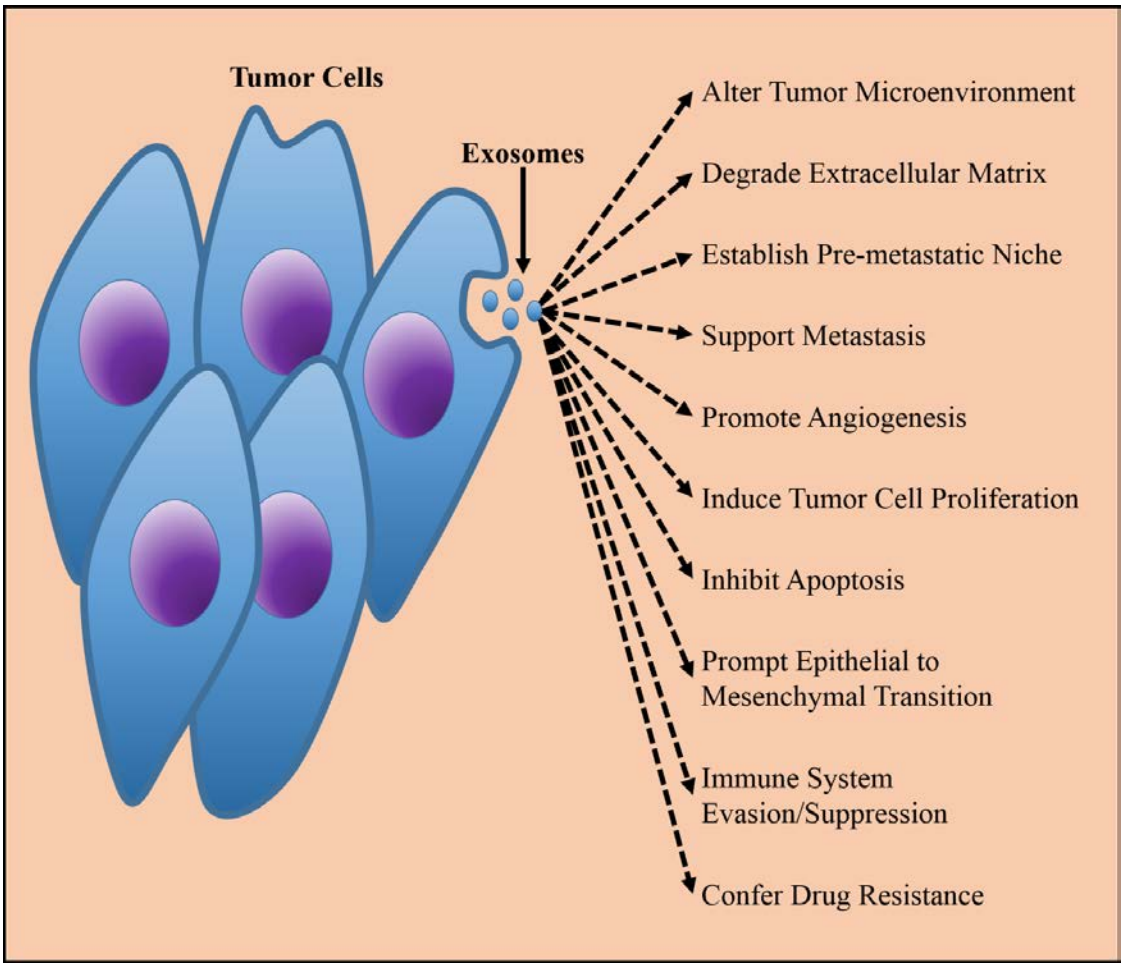

Figure 2. Tumor derived exosomes are released constitutively from cancer cells, and are capable of relaying information which reprograms target cells and modifies physiological environments in miens beneficial to cancerous growth and metastasis.

Communication with the tumor microenvironment is vital for tumor progression and metastasis. Cancer cells secrete exosomes to reprogram their environs and establish favorable conditions for tumor growth and invasion of healthy tissues. This microenvironment is comprised of the ECM and stromal cells including fibroblasts,

endothelial and inflammatory immune cells, and tumor-associated vasculature (72). Evidence is also accruing that links adipose stromal cells, or adipocytes, to promoting the tumorigenic microenvironment, especially in obesity-related cancers (73). Fibroblasts synthesize ECM and are essential to repaving this extracellular network during aberrant cell growth. Cancer exosomes can induce fibroblasts to become more activated in laying the framework for this favorable tumor microenvironment by eliciting the TGFβ/Smad pathway in target fibroblasts (74). Fibroblast remodeling of the tumor microenvironment can also be promoted by the exosomal secretion of ECM metalloproteinases from tumor cells (75).

Epithelial-to-mesenchymal transition, a hallmark of tumor microenvironments becoming more aggressive and metastatic, can only be accomplished through intercellular communication and evidence recently was reported that tumor exosomes are a contributing factor (76). This process of EMT is led by oncogenic transmission that is possibly mediated by exosomal cargo transfer which modulates certain aspects of differentiation associated with tumor-driving EMT (77).

Brain tumor cells expressing an oncogenic epidermal growth factor receptor (EGFR) were shown to export and deliver this mutant EGFR to other cells, thus transferring oncogenic activity leading to activation of MAPK and Akt signaling pathways, morphological transformation, and anchorage-independent growth (78). Such alterations can lead to consequent production of angiogenic factors such as vascular endothelial growth factor (VEGF) which can facilitate vascularization to the tumor mass.

Cancer exosomes are clearly powerful mediators with the aptitude for changing the behavior of neighboring cells. This becomes even more evident with their ability to promote the formation of the pre-metastatic niche. For metastasis to occur, not only do cancer cells need to migrate to a new environment, but that environment must be conditioned appropriately to allow colonization.

An elegant *in vitro* and *in vivo* experiment demonstrated that exosome secretion was required for direction cell movement and persistent migration of cancer cells (79). The experiments by Sung et. al utilized live-cell imaging to show that exosome secretion directly preceded and enabled adhesion assembly via an exosome induced autocrine signaling with fibronectin housed inside exosomes as the critical component. Therefore, cancer exosomes are capable of secreting and delivering necessary ECM molecules to modulate integrin and adhesion formation to drive the migration and invasion of cancer cells.

It has been illustrated that exosomes derived from metastatic melanomas promoted metastatic behavior of primary tumors through the horizontal transfer of MET oncoprotein to bone marrow progenitor cells, a process referred to as “educating” for metastatic colonization (80).

Liver pre-metastatic niche formation was shown to be induced from pancreatic ductal adenocarcinomas (PDAC) derived exosomes that expressed high levels of macrophage migration inhibitory factor (MIF) and led to a fibrotic-microenvironment. Via a MIF-blockade, liver pre-metastatic niche formation was prevented, and upon measuring

exosomal MIF levels in patients with stage-1 PDAC, those with higher levels later developed liver metastasis compared to those with low MIF levels (81).

Intra-vital imaging of cancer exosome uptake by non-cancer cells using the Cre-LoxP system, showed that mRNA cargo delivered to non-malignant cells induced enhanced migratory potential and metastatic capacity (82).

In addition, miRNAs have an intriguing role in cancer and exosomes. MiRNAs are non-randomly added to exosomes and carry functional information from cancer cells which can phenotypically change target cells in a fashion that shapes and alters microenvironments to allow favorability to cancer cell growth and invasion (83). Melo et. al. showed that miRNA maturation occurred in exosomes after their incorporation into vesicles. When compared to miRNA content of exosomes derived from healthy cells, the cancer exosomes had a disproportionately higher concentration of mature miRNAs. This suggests that cancer exosomes might not only act as simple postage boxes, but are rather active facilitators in the processing of their own cargo. Breast cancer exosomes with functional miRNAs are capable of altering target cell transcriptomes and instigating non-cancer cells to become more tumorigenic (84). The miRNA family, miR-200, regulates the process of EMT, mentioned above, and was seen to be increased in serum exosomes of cancer patients (85). Transfer of miR-200 via cancer exosomes, therefore, increased the metastatic potential of target cells by altering gene expression to favor EMT.

A recent study presented that a series of tumor cell lines all secreted exosomes containing the inhibitors of apoptosis (IAPs) Survivin, cIAP1, cIAP2, and XIAP. The authors suggested that cancer exosomes contain these IAPs as a possible warning signal or

as an added layer of protection to the rogue proliferating cells from an ever-changing tumor microenvironment (86).

The effects of cancer exosomes on the immune system is two-handed, as they can induce immunosuppressive functions that uphold tumorigenesis or can provide a boost to the immune response to tumors. Apoptosis of CD8+ T cells can be induced by cancer exosomes through the death receptor pathway (87). Cancer exosomes can lead to further T cell dysregulation by inducing the proliferation of regulatory T cells and inhibit effector T cell proliferation (88). Additionally, cancer exosomes can negate the cytotoxic functions of natural killer cells (89).

On the other hand, cancer exosomes can spread antigens, increasing dendritic cell presentation of those antigens. Also, exosomes can interact with memory T cells leading to antigen-specific immune responses against the tumors (90).

Cancer exosomes are also implicated in tumor resistance to chemotherapeutic drugs. The removal of cisplatin and trastuzamab from cancer cells by exosomes indicates a drug-scavenging function (88). It was also shown that certain chemoresistant cancer cells could horizontally transmit their drug-resistant phenotypes through their exosomal miRNAs (91), and an increasing number of studies are linking exosomal miRNAs to the ability of cancer cells to acquire drug resistance and conduct that resistance to other cancer cells (92). Mesenchymal stem cells (MSC) are known to be involved in chemotherapeutic drug resistance and MSC-exosomes have been implicated in promoting drug resistance in gastric cancer by activating the calcium/calmodulin-dependent protein kinase (CaM-Ks) and Raf/MEK/ERK kinase cascade (93).

A recent review by Braicu et. al outlines even further how secreted messages from cancer-derived exosomes use both membrane and cytosolic constituents, particularly miRNAs, to act as critical components of the tumorigenic circuit that disrupts the normal condition of healthy cells into the development of oncogenesis (92, 94).

Exosomes in Therapy

Unsurprisingly, due to their strong implications in cancer pathogenesis and biological compatibilities (i.e. their ability to cross physiological barriers like the blood brain barrier), exosomes are strong candidates for myriad therapeutic applications. These possibilities include targeting exosomes that appear to be progenitors in cancer progression, engineering exosomes as therapeutic devices, and discovering novel biomarkers for early diagnosis and identifying molecular targets. Aside from cancer, beneficial effects of therapeutic exosomes have already shown promise in myocardial ischemia reperfusion and kidney injury (95), myocardial infarctions (96), muscle or bone regeneration (97), arthritis (98), nerve regeneration (99), multiple sclerosis (100), and neurodegenerative diseases such as Alzeihmer's or Parkinson's (101).

Due to their selective cargo loading and resemblance of their producer cells, exosomes are valuable for discovering cancer biomarkers (Figure 3). With increasingly improving isolation techniques from cell culture and patient blood, and methodology for characterizing cancer exosome components, scientists are utilizing exosomes to identify molecules to target cancer more effectively and apply more personized techniques to detection, diagnosis, and prognosis. Protein characterization by mass spectrometry (59), as well as immunocapture techniques for identifying and quantifying peptide and nucleic

acid (miRNA, mRNA, etc.) profiles (102) and commercially available products already provide useful approaches to biomarker discovery. Some of the most recent cancer exosome biomarker studies include complete proteome analysis of melanoma exosomes (103) and circulating biomarkers (104), miRNA biomarker analysis of esophageal adenocarcinoma (105), prostate cancer (106), glioblastoma (107), serum miRNAs for acute myeloid leukemia (108), colorectal cancer (109, 110), gastric cancer (111), urinary exosomal miRNAs for ovarian cancer (112),[113], pancreatic cancer specific proteoglycan (114), proteomic biomarker profiling of cholangiocarcinoma (115), non-small cell lung cancer (116), glioma (117), and salivary exosomes for oral cancer (118). In addition, it has been discovered that circular RNAs (circRNA) are stably expressed in exosomes and these circRNAs are suggested to be a promising candidate for biomarkers in cancer(119). These examples provide insight that exosomes can be used as a more sensitive and less invasive technique to cancer diagnostics.

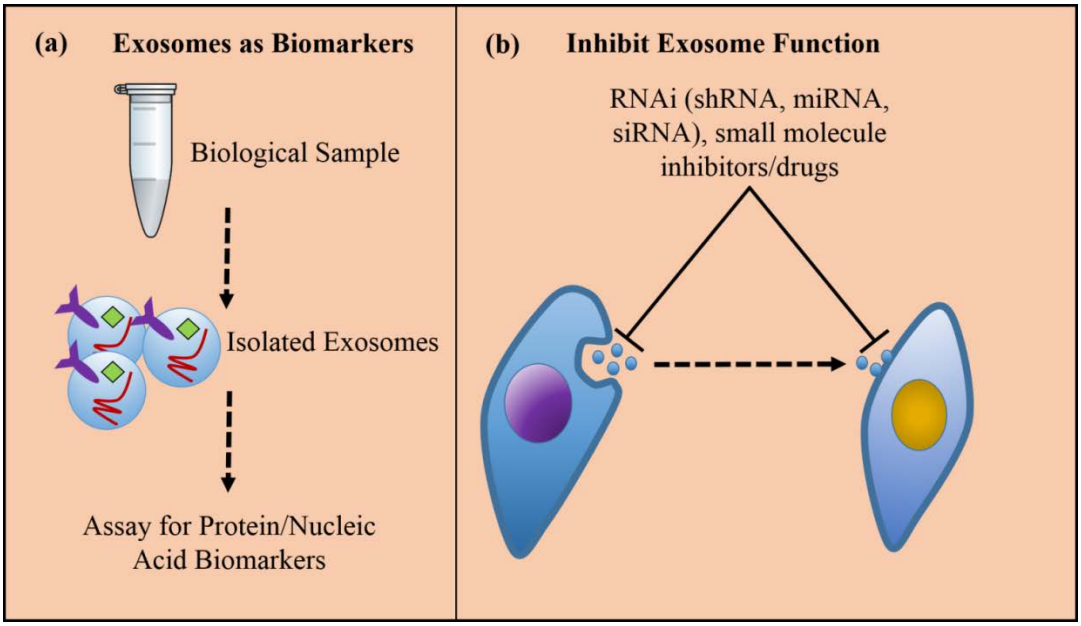

Figure 3. (**a**) Exosomes can be isolated from cell culture supernatants or patients' bio-fluids to assign diagnostic and prognostic signatures of cancer by profiling exosomal proteins or RNAs, therefore exosomes potentiate a non-invasive, or liquid biopsy, technique for assessing tumorigenesis and cancer progression. (**b**) Inhibiting exosome function is one particular therapeutic strategy for pacifying the cancer promoting effects of tumor-derived exosomes either by blocking the formation and release of the exosomes from the producer cell, preventing uptake of the exosomes in the target cell.

Very recently, the cell surface proteoglycan, glypican-1, was identified as being specifically enriched on cancer exosomes. Monitoring glypican-1 on circulating exosomes demonstrated specificity and sensitivity in distinguishing between healthy subjects and patients with benign pancreatic cancer from early/late stage pancreatic cancer patients (114). Glypican-1 on circulating exosomes may be an efficient non-invasive screening tool for pancreatic cancer, and exemplifies the possibilities of exosomes for cancer diagnostics.

Attenuating the production and release of exosomes from tumor cells is one important therapeutic paradigm given that circulating exosomes nearly double in cancer patients and their cargo promote tumor progression and spread (Figure 3).

One such method would be to inhibit certain molecules that are required for exosome formation within the cell (i.e. the endosomal pathway) such as ceramide synthesis via the sphingomyelinase pathway. The use of amiloride to reduce exosome production and reduce tumor progression was observed *in vivo* via myeloid-derived suppressor cells which suppress T cell activation (120), but similar results were not seen with amiloride treatment

of prostate cancer cells (121) suggesting that this mode of inhibition is cell-type dependent. Other factors that are involved in exosome biogenesis such as the ESCRT pathway and the syndecan proteoglycan and adaptor syntenin are possible targets also.

The application of RNAi to inhibit certain gene regulation is of particular interest as their mechanistics are becoming better understood, the design of functional small interfering RNAs (siRNA) is improving to the point of preclinical and clinical trials (122). RNAi and small molecule inhibition of targeted exosome biogenesis molecules can effectively knockdown certain production characteristics of exosomes and be utilized for preventing for preventing exosome dissemination from diseased cells which might in turn lead to spread of disease phenotypes to target cells. The mechanisms for this action are either by gene knockdown by RNAi, such as engineered shRNAs that bind to, and prevent translation of exosome-production machinery including ESCRT proteins and/or GTPases involved in producing exosomes (Vader et. al) (123). For example, targeting the GTPase RAB27a which is required for the release of some tumor exosomes. Peinado et. al demonstrated in their experiments mentioned above that RNAi of Rab27A GTPase in melanoma cells greatly abrogated exosome production and bone marrow education, consequently reducing the metastatic potential of the cancer (80). Another study in mammary carcinoma cells led to decreased primary tumor growth and lung dissemination upon a blockade of RAB27a (124). In addition, other GTPases that serve as factors in the docking/fusion of the MVB to the plasma membrane can serve as potential targets for deregulating exosome secretion from tumor cells.

Sung et. al illustrated in their experiments that knockdown of Rab27a and Syt7 reduced cancer exosome secretion between 2.2 and 3 fold fewer compared to normal cells and also dysregulated cell polarization and migratory persistence (79).

Another possible target for inhibiting the tumorigenic function of cancer exosomes is to prevent the fusion or uptake of exosomes by target cells. One experiment prevented tumor-derived exosome uptake by cells through blocking phosphatidylserine with diannexin (125).

It should be noted, however, that this mode of repealing exosome function poses potential complications in that many normal physiological processes might be inadvertently afflicted.

An evolving approach in therapeutic exosomes is using them as drug delivery devices. Exosomes are ideal vehicles for molecule delivery (proteins, RNAs, small molecule drugs/drug oligonucleotides, etc.), due to their biocompatibility, stability in circulation, and ability to target them to certain cell types. Small interfering RNAs (siRNA) have enormous potential as therapy with their gene-knockdown effects, but are difficult to employ due to their high instability. Exosomes provide an innovative and newly popular device for carrying siRNAs, as well as shRNAs, miRNAs, and mRNAs. The expression profile of tumor exosome miRNAs becomes dysregulated in many cancers and can be used for tumor characterization and diagnostics, as well as therapeutic payload (126). One study exemplified this aspect by loading MSC-exosomes with miR-146b and managed to reduce primary brain tumor growth in rat glioma by intra-tumoral injection (127). Elucidating the

natural mechanisms of miRNA loading into exosomes is imperative to progressing the use of miRNA as therapeutic cargo.

Drug loading can be accomplished either endogenously or exogenously (Figure 4). Endogenous, or passive, loading is carried out by overexpressing the RNA species or molecule of interest in producer cells. This passive loading is enabled by the cell's native exosomal loading mechanisms and results in exosomes that contain the drug prior to isolation. Exogenous, or active, loading begins with exosome collection and requires either co-incubation or electroporation of the exosomes with the drug/molecule of interest (55). Theoretically, it is possible that one could use exogenous drug loading on previously endogenously loaded or engineered exosomes as a more wide-ranging tactic to this methodology.

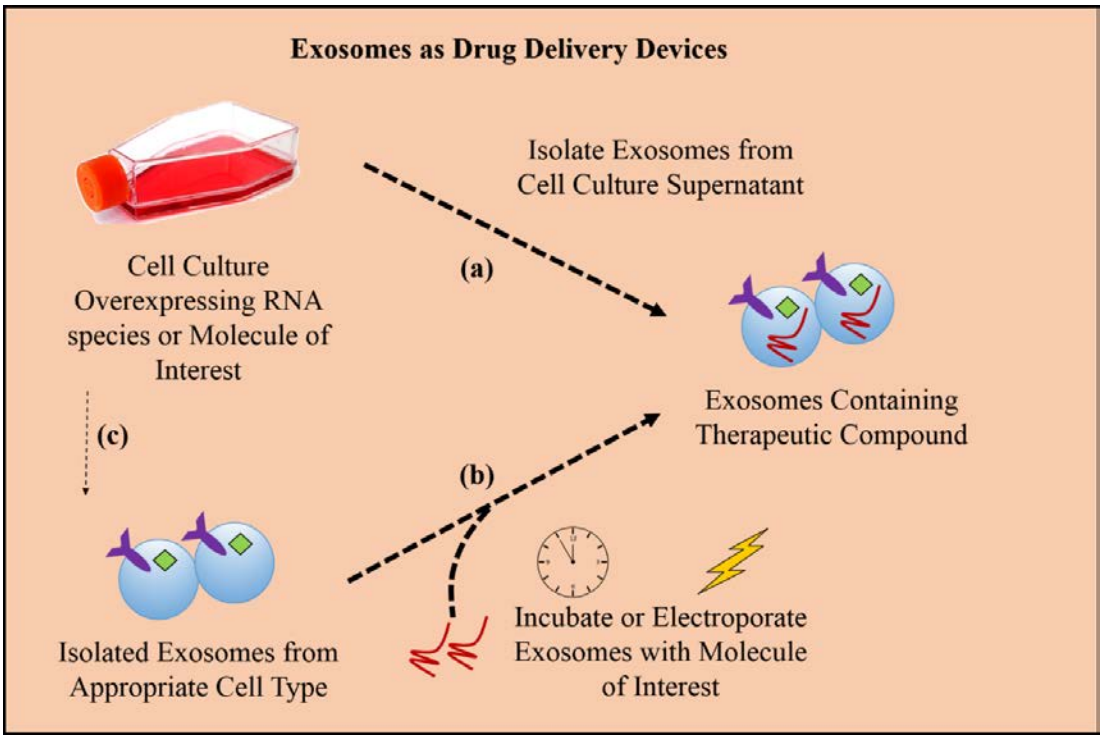

Figure 4. Loading exosomes with therapeutic cargo, such as RNA species for gene knockdown in targeted cancer cells or small molecule drugs of interest, can be achieved in two ways: (**a**) endogenously, by collecting exosomes from cells overexpressing the molecule of interest, or (**b**) exogenously, by collecting exosomes from an appropriate cell culture that produces exosomes suitable for specific targeting and then incubating or electroporating the exosomes with the molecule of interest. Once the exosomes are successfully loaded, they can be used for downstream therapeutic applications. (**c**) Additionally, it is theoretically possible to combine the two methodologies as a more comprehensive approach to loading with molecules into pre-engineered exosomes.

Additionally, it may be possible to use viral packaging strategies to load exosomes with molecules (128, 129) and marketed kits have become available to load exosomes in culture with proteins of interest, for example the XPack technology from System BioSciences (https://www.systembio.com/xpack)..

Exosomes targeted to specific cell and tissue types can enhance specific uptake and reduce off-target deliveries. Cell or tissue targeting can be achieved by engineering exosomes to express plasmid fusion constructs with targeting ligands fused to extracellular membrane proteins. For example, exosomes were collected from mouse immature dendritic cells engineered to express Lamp2b fused to a tumor targeting integrin and loaded with doxorubicin by electroporation. Intravenous injection of the engineered exosomes

delivered doxorubicin specifically to the specific integrin-positive breast cancer cells leading to inhibition of tumor growth whereas untargeted exosomes localized to the liver and spleen (130). Different investigations showed that brain endothelial cell derived exosomes are successful at crossing the blood brain barrier to deliver anti-cancer drugs in a brain cancer zebrafish model (131) and that intra-tumoral injection of exosomes engineered to express an anti-tumor miRNA reduced glioma growth in rat models(127).

Choosing the correct cell line for therapeutic exosome production is important for a few reasons. The exosome must be lacking in immune-stimulating activity to prevent unwanted immune effects in target tissues. For this immunogenic reason, immature dendritic cells have been favorable choices (132). Cell choice can also dictate the native population of exosomal surface proteins that might have a desirable ligand-receptor interaction with the proposed target cell. Finding this optimal producer-target cell combination is vital to producing exosomes for therapy. There is also the opportunity to create entirely artificial exosomes with therapeutic cargo and ideal surface moieties for target cell specificity. Strategic advances are being made in producing exosomes with targeted peptides via glycosylation sites for enhanced targeted delivery of exosomes for therapeutics (133).

Tracking exosomes *in vivo* after injection is becoming more apparent in the literature and methodologies better established using fluorescent labels or membrane dyes (80, 128, 134, 135). These technologies allows researchers to resolve the biodistribution and local enrichment of injected exosomes. Tracing the transfer of functional exosomal cargo, such as RNAs, within the tumor microenvironment *in vivo* can provide researchers with the identities of possible targeting sites for anti-cancer drugs and engineered exosomes (136).

Human MSC-exosomes, which have intrinsic therapeutic activity, appear to be promising producers of exosomes for therapeutic applications and drug delivery as they are known to have successful therapeutic benefits in diseased animal models and display immunosuppressive activity (137). MSC-exosomes delivered to mouse breast cancer cells delivered molecules which led to the downregulation of VEGF and therefore decreasing tumor growth by suppressing angiogenesis (138).

The role of MSC-exosomes in promoting drug resistance in gastric cancer, as mentioned in the previous section, can be inhibited by blocking the CaM-Ks/Raf/MEK/ERK kinase cascade (93).

Exosomes have potential applications as cancer vaccinations as well. Exosomes loaded with α-galactosylcerimide and tumor specific antigen can activate cancer-specific adaptive immune responses decreasing tumor growth (139), and separately, isolated tumor-derived exosomes carrying tumor antigen were shown to effectively induce anti-tumor immune responses in primary and metastatic mouse melanoma models (140).

Conclusions

The idea of improving healthcare through personalized medicine is a growing field. Personalized medicine designates that tumor treatment be molded to the individual's characteristics, biological signatures, and response to specific treatment. Hence, exosomes hold a spot in the development of efficacious personalized therapeutic techniques given their use for biomarker discovery and personalized diagnostic capacities. In the future, it

might also be possible to isolate circulating exosomes from an individual, or from specifically harvested cell types, load them with specific molecules, *in vitro* with techniques mentioned above, tailored to a specific therapeutic strategy, and redeliver the modified exosomes back to a patient to induce a relevant response (i.e. reduce tumor growth).

Disease intervention with exosomes is an exciting new avenue in therapeutics with novel strategies for cancer treatment. There is promising evidence supporting the use of exosomes as diagnostic tools for discovering biomarkers, targeting exosomes to inhibit their disease related functions, exploiting them as drug delivery devices, and utilizing their inherent therapeutic potentials. Further investigation is required to drive exosome based therapeutics to the next level of research and eventual clinical trials that will clarify the complex aspects of exosomes that both promote and mollify malignant environments.

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Author Contributions

Phillip Munson wrote and created the artwork under supervision of Arti Shukla. Manuscript was reviewed by both authors.

Conflicts of Interest

The authors declare no conflict of interest.

Introduction to Exosomes and Cancer

Book chapter "Introduction to Exosomes and Cancer" in the title "Diagnostic and Therapeutic Applications of Exosomes in Cancer". Eds: Mansoor M. Amiji and Rajagopal Ramesh. Elsevier. May 2018.

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Abstract

Cancer research has found a novel foothold in studying exosomes, the 40-140nm membrane bound vesicles secreted by cells as molecular messengers. These secreted vesicles of endocytic origin act as signaling conveyors between cells by shuttling molecular cargo in the form of proteins, mRNA, miRNA, and lipids. The many roles of exosomes in normal physiology and disease are becoming clearer as they are increasingly studied. Their role in cancer is being found to range from sending pro-tumorigenic messages between cancer cells and to non-cancer cells to aid in the growth and spread of the tumor. Tumor exosomes are implicated in angiogenesis, metastasis, drug resistance, immune evasion, and even more processes involved in the pathophysiology of cancer. As we begin to uncover these roles, researchers are discovering the importance of understanding exosomes, as they pertain to cancer, as a means of discovering much needed biomarkers, elucidating the mechanisms of cancer biology, identifying therapeutic targets, and using exosomes themselves as a mode of therapy against cancer.

Keywords: exosome, cancer, extracellular vesicle, biomarker, intercellular communication

A Brief History

One of the most exciting and cutting edge topics in modern day science orbits the now dominant theme of extracellular vesicle research. Of particular interest, is in regards to the subset of extracellular vesicles (EVs) referred to as exosomes, which can be seen by the rapid increase of publications over the past 30 years (Figure 5). Cell-derived vesicles first arrived on the scientific radar in the 1940's when cell-free plasma was discovered to contain a clot-inducing subcellular element (141). Decades later the term 'platelet dust' was used to describe 20-50nm vesicles, followed the usage of the term microvesicles in 1975 (142, 143). By 1981, the coinage, "exosome," was used by Trams et. al regarding exfoliated vesicles with 5'-nucleotidase activity composed of increased amounts of sphingomyelin and polyunsaturated fatty acids (49). Subsequent discoveries over the last decades provided a more comprehensive understanding of the characterization, origin, biogenesis, and functions, which will be briefly summarized in this chapter before linking the ongoing trajectory of exosomes in regards to cancer research.

Currently, the term exosome refers to small (40-140nm) membrane bound vesicles of endocytic origin and are not to be confused with the larger (200-1,000nm) microvesicles produced by direct shedding from the plasma membrane. Traditionally, exosomes were deemed as nothing more than a waste mechanism for cells to dispose of unwanted material. However, it has become evidently clear that exosomes serve a much more biologically

sophisticated purpose in relaying messages between cells and tissues. Such molecular messages are profoundly adept at altering target cell phenotypes, as scientists have come to discover. Exosomes are known to be present in nearly every body fluid sampled from blood, cerebral spinal fluid, urine, lymph, amniotic fluid etc., and all mammalian cell types appear to be capable of producing these vesicles. Once released into the extracellular space, exosomes can travel to sites near, or distant from their dissemination, thereby providing potential for endocrine, paracrine, and even autocrine signaling. The molecular content of exosomes is of notable intrigue, ranging from biofunctional proteins, RNA species (mRNA and particularly microRNAs), lipids, and some reports indicate the presence of genomic DNA (50).

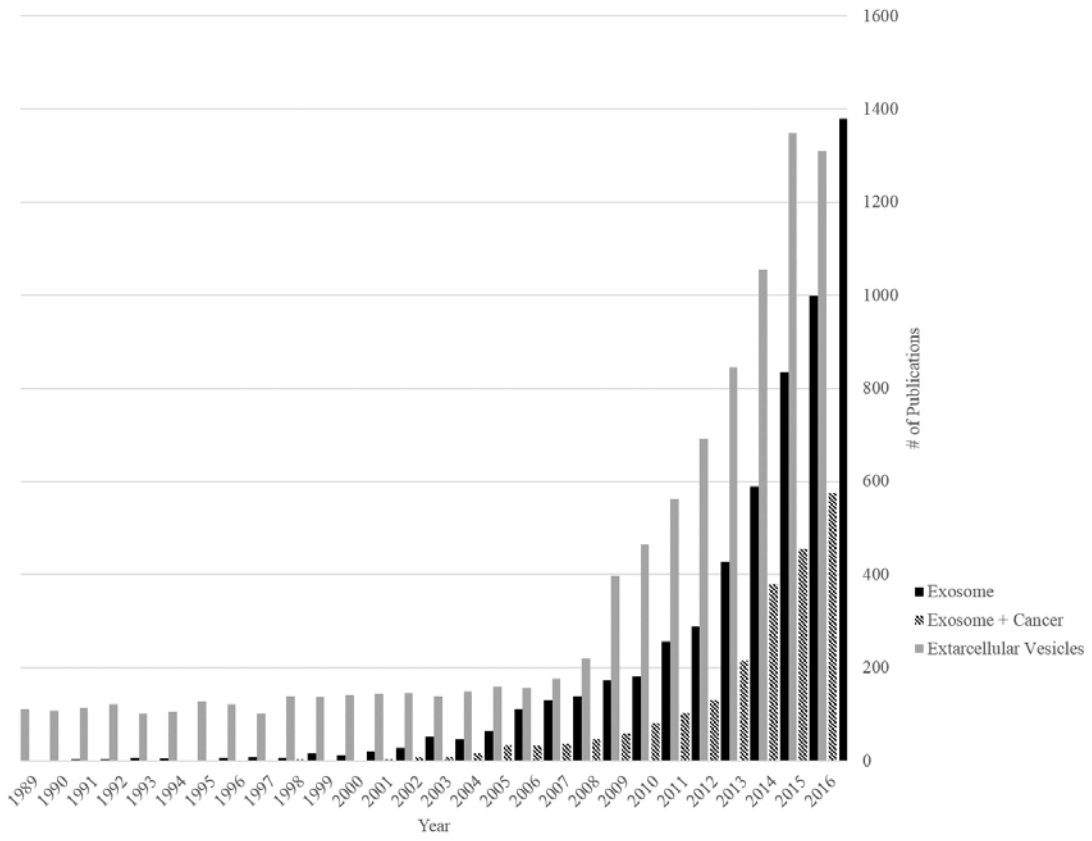

Figure 5. Number of publications listed on PubMed using the keywords “exosome,” “exosome cancer,”, or “extracellular vesicles.”

Biogenesis

The biogenesis of exosomes, as mentioned above, is of endocytic origin and involves a more complicated pathway than that of microvesicles. After the primary invagination of the cell membrane through endocytosis, exosomes begin their creation on the surface of the endosome. A secondary invagination occurs here leading to the deposition of smaller vesicles inside the endosome. These smaller vesicles, at this stage, are referred to as intraluminary vesicles (ILVs), and the endosome containing them is now referred to as either a mature endosome or a multivesicular body (MVB). There are two fates for the

ILVs at this stage, one is to be targeted for degradation by the lysosome, which appears to be regulated by lipid composition, cholesterol poor membranes, and/or the presence of lysobisphosphatidic acid (68). The other fate for the MVB, and its content ILVs, is to proceed to fusion with the producer cell's internal surface of the plasma membrane, thereby releasing the contents to the extracellular space. The now released vesicles are exosomes.

The mechanisms by which ILVs/exosome are produced during the secondary invagination on the endosomal membrane is yet to be fully understood, but certain components have been identified. One mode of exosome biogenesis requires the endosomal sorting complex required for transport (ESCRT) and is known as the ESCRT-dependent pathway, whereas there is also an ESCRT-independent pathway. The ESCRT-dependent pathway utilizes the accessory protein ALIX for sorting of syndecans through syntenin-mediated interactions and is comprised of four separate complexes: ESCRT-0 for loading ubiquinated proteins onto the endosomal surface; ESCRT-I and ESCRT-II for budding of the endosomal membrane; and ESCRT-III for separating the membrane. The ESCRT-independent pathway is reported as involving and requiring the lipids sphingosine-1 phosphate and ceramide, the enzyme sphingomyelinase, and tetraspanin enriched microdomains (56, 144) . Both of these pathways are targets for inhibition of exosome secretion.

During the process of exosome genesis, lipids are sorted at the site of invagination and molecular cargo is packaged. The exact mechanisms of this process are also unclear, but it is known that there are four underlying requirements for this to occur: cytoskeletal components (actin, microtubules etc.), molecular motors (kinesin, myosin), molecular

switches (predominantly GTPases), and fusion machinery/tethering factors such as SNAREs (57). Interestingly, exosomes apparently have a diverse range of functions depending on the mode by which they are generated (58, 145).

Once generated within the MVB, exosome release is mediated by small vesicular transport regulation GTPases (Rab27A, Rab11, and Rab31), which can work with SNAREs to fuse the MVB membrane to the internal surface of the plasma membrane, and these components are another area of interest for inhibiting exosome secretion (66).

Isolation and Characterization

In order to adequately study populations of exosomes, researchers are working toward standardizing isolation techniques. However, more and more techniques are being introduced that make a standard approach increasingly unlikely. Nevertheless, the traditional gold-standard for exosome purification is differential ultracentrifugation and most commonly, exosomes are isolated from conditioned cell culture supernatant supplemented with exosome-free fetal bovine serum, but exosomes are also commonly collected from bodily fluids like plasma or urine. This technique is widely used because it is less likely to have contaminate protein aggregates, however it is highly time intensive and requires high volumes of media, as well as yielding low amounts of pelleted exosomes (146). Before ultracentrifugation, samples are cleared of cell and cell debris by shorter, lower speed spins followed by 100,000 to 150,000 × g spins for about 1 hour to clean the sample prior to another high speed spin. Another common technique applauded for clean sample prep is density gradient centrifugation which utilizes sucrose cushion to separate out vesicles based on size, mass, and density (147, 148).

In addition, exosomes can be captured using various ultrafiltration techniques or size-exclusion chromatography which have become commercialized to separate preps based on size and molecular weight (149, 150). Immuno-affinity capture techniques are being employed to separate out exosomes based on their surface protein markers such as CD63, CD81, and CD9. Another popular method is by exosome precipitation, where samples are cleared of cells/debris and typically a solution of polyethylene glycols is added to insolubilize small exosome-sized vesicles and after an overnight incubation can be spun out at low speed (151, 152).

There are multiple means, after isolation, by which to characterize exosome preparations. The most agreed upon standards for defining an exosome now are membrane bound vesicles with a diameter of 40-140nm coming from endocytic origin. Because of this, size characterization and Western blot analysis for exosome markers such as certain tetraspanins (CD9, CD63, and CD81), MVB proteins (ALIX and TSG101), and heat shock proteins ((Hsc70, Hsp 90) are most commonly employed (153). Size characterization includes multiple avenues, most useful of which is nanoparticle tracking analysis, which not only provides size characterization, but also particle concentration and, in many circumstances, zeta potential. Dynamic light scattering can be used to determine exosome size populations, but to less effectiveness as nanoparticle tracking analysis. Additionally, transmission electron microscopy (TEM) can allow size characterization along with morphological characterization that is useful for defining pure exosome isolation, and recently scanning electron microscopy has been employed (154). TEM allows visualization of the double membrane structure of exosomes, along with a notable cup-shaped

morphology that is indicative of exosomes in TEM. Additional techniques are also being developed and used, such as high-resolution flow cytometry (155), and microfluidic-based systems (156).

Content

Exosomes have been reported to be released from nearly every cell type and bodily fluid studied, and can interact with myriad target cell/tissue types. The molecular cargo of these exosomes dictate the diverse range of effects they may have on target cells, either near or far from their production. Such content ranges from proteins (surface or cytoplasmic), RNA species, functional lipids, and occasionally reported genomic DNA (157, 158). The surface protein content of exosomes resembles the surface composition and topology of its producer cell, and these surface molecules are determinates for potential target cell interaction, and such interactions are pertinent to normal physiology as well as disease processes (159). It should be noted here that there are multiple ways in which an exosomes may interact with a target cell: docking to cell surface protein and conducting a signaling cascade within the cell; the exosome may be endocytosed; or there may be direct fusion of the exosome to the target cell membrane.

Beyond proteins, RNA species enriched in exosomes are of robust functional importance to the capacity of exosomes to elicit a biological effect. There have been many RNAs identified in exosomes to date (mRNA, piRNA, snoRNA, scaRNA, Y RNA, siRNA, tRNA fragments, vault RNA), but having gained the most attention is undoubtedly miRNAs (64).

The focus on miRNAs is because these small (around 22 nucleotides in length) non-coding RNA strands are now known to regulate up to half of the genes within the human

body (91), and are highly enriched in exosomes. These miRNAs are transferred to target cells via exosomes, maintaining functionality to post-transcriptionally regulate gene expression, and are therefore of great interest when studying exosome components and signaling.

Biological Function in Cancer

The many roles of exosomes in normal physiology is vast: immune function and surveillance; neural plasticity and brain function; tissue repair; stem cell maintenance and function; blood coagulation; heart function and cardio protection; and the list could go on (54, 160). Conversely, exosomes also play an outsize role in many disease states including: pathogenesis of viruses like HIV-1and parasites like malaria (161, 162); heart diseases (163); kidney diseases, diabetes, and metabolic disorders (164, 165); disorders of pregnancy (166); central nervous system diseases such as Parkinson's, Alzheimer's, and multiple sclerosis (167, 168); and, as this book will focus on, exosomes are enormously important in the biology of cancer (70).

Because these small vesicles are such big players in human physiology and cancer, it is fundamental that researchers use them to uncover the mechanisms of healthy and abnormal physiologies, while also utilizing the molecular cargo as quarries of biomarkers. The dynamic role of exosomes secreted by cancer cells is substantial and are becoming understood to be involved in tumorigenesis, tumor growth/progression, metastasis, angiogenesis, extracellular matrix (ECM) remodeling, immune evasion, chemoresistance, and the establishment of the premetastatic niche (55, 80). On the front of biomarkers, it is important to note that neoplastic cells secrete exosomes with content that is markedly

dissimilar to that of their non-cancerous counterparts, and in many cases it has been reported that cancer cells secrete a larger volume of exosomes altogether when compared to non-cancerous cells. The unique signature associated with cancer exosomes is an exciting front in excavating for biomarkers to diagnose cancer, provide better prognostics, and identify therapeutic targets.

Proteomic identification and analysis of miRNAs as exosomal biomarkers of cancer is of great interest and importance to the field. Protein profiling from exosomes has led to very intriguing finds that may lead to clinical use, such as the discovery that Glypican-1 in exosomes can identify pancreatic cancer (114). Differential expression of miRNAs in exosomes, not only provides insight for biomarkers (169), but the array of functionality imparted by transferred exosomal miRNAs to tumor cells function phenotypically change target cells (170). Interestingly, along with sending pro-tumor miRNAs to targets, it is being uncovered that some tumors even shuttle tumor suppressor miRNAs away from themselves via exosomes to prevent their antitumorigenic effects (171, 172). Identifying such biomarkers and their mechanistic effects is of utmost importance in the realm of understanding cancer and the field of exosomes has significant potential.

Tumor derived exosomes carry their epigenetic cargo to other tumor cells to aid in their progression and also to non-tumor cells for the purpose of phenotypically altering them in order to aid in tumor growth and spread. The alterations caused by tumor exosomes to non-tumor cells can be that they dampen the immune response against the malignancy, reprogram surrounding cells in the tumor microenvironment to aid the tumor, or even convert non-malignant cells to become cancerous. The established communicatory link

between tumor cells and host cells via exosomes turns out to be dynamic system that promotes tumor survival. Tumor exosomes are capable to directly target immune cells to aid in the tumor's evasion from the immune system by carrying immuno-inhibitory signals to immune cells (173). In addition, tumor exosomes are adept in establishing cells in the tumor microenvironment to make the location more favorable for the tumor. The cells that can be targeted in this setting include fibroblasts, stromal cells, endothelial and other inflammatory immune cells, and the vasculature surrounding the tumor. Altering and repaving the framework of the ECM by this route is accomplished by exosome signals to these cells and also by exosomal secretion of ECM metalloproteinases (72, 74, 75). Further, tumor exosomes are reportedly capable of leading to the well-characterized herald of tumorigenesis known as epithelial to mesenchymal transition, suggesting an extra layer of exosomes' role in coordinating the spread of cancer (76). Exosomes promote angiogenesis and also are capable of conferring chemotherapeutic drug resistance to cancers by allowing the transfer of genetic cargo that more quickly allow the tumor cells to adapt and become resistant, but the exosomes are also used by the cancer cells to spit out the chemotherapeutics that are internalized (174, 175).

The therapeutic approaches using exosomes and what is newly being discovered is expanding greatly. Not only are the molecules present in tumor exosomes useful as therapeutic targets, but exosomes themselves can be engineered as therapeutic delivery agents and other treatment approaches include the inhibition of exosome secretion from tumor cells. The therapeutic potentials for exosomes in cancer include the direct targeting of exosomes that tumor cells produce and may be the progenitors of its progression, using

them as drug delivery devices, and by using them to as diagnostic and prognostic indicators of tumorigenesis based on biomarker discovery (176-180).

As the field of cancer exosomes expands, we are likely to uncover fascinating insight into the biology of cancer and the sophisticated mechanisms by which cancer develops, grows, and spreads. In these efforts, it is becoming clear that exosomes have enormous potential to biomarker discovery and therapeutic options that will shift the paradigm by which we understand, diagnose, and treat cancer.

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1.4 Exosomes in MM

Potential roles of exosomes in mesothelioma development and diagnosis: where are we?

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Abstract

Malignant mesothelioma (MM) is a devastating cancer of mesothelial cells, caused by asbestos exposure. Limited knowledge regarding the detection of asbestos exposure and early diagnosis of MM, as well as lack of successful treatment options for this deadly cancer, project an immediate need to understand the mechanism(s) of MM development. With the recent discovery of nano-vesicles, exosomes, with enormous potential to contain signature molecules representative of different diseases as well as to communicate with distant targets, we were encouraged to explore their role(s) in MM biology. In this review we summarize what we know so far about exosomes and MM based on our own studies and published literature from other groups in the field.

Key words: Exosomes, malignant mesothelioma, asbestos

Introduction

Few areas of research have grown as quickly and with as much enthusiasm as that of extracellular vesicle research related to exosomes. Exosomes are small, 40-140nm membrane bound vesicles secreted from cells and originating from the endosomal pathway. These vesicles are enriched in biologically functional molecules (proteins, mRNA, miRNA, DNA and lipids) and are vital to intercellular communication (51). The communication conduit established by exosome transport from producer cells to target cells is important to normal physiology as well as disease states, such as cancer (181).

The stampede of studies in the field of exosomes has flooded valuable information about basic biology and disease into the scientific sphere. We now know that exosomes are more than simple waste receptacles used by cells to rid themselves of unwanted material, but are sophisticated molecular messaging systems that can act locally or distal from where the vesicles are secreted. Exosomal communication is implicated in a myriad biological systems from immune function and tissue repair (182), nervous system signaling (183), cardiac health (95, 184), to more sinister roles in viral pathogeneses like HIV-1 (185),

Due to the pivotal roles exosomes play in disease, they provide much needed insight into progressing research into avenues such as biomarker identification for diagnostic and prognostic means (186), as well as identifying disease mechanisms as therapeutic targets (178, 179).

The term asbestos refers to a group of hydrated silica fibers that occur naturally throughout the world. Classified as a category 1 carcinogen (187), asbestos is one of the more notoriously well-known cancer causing agents. Derived from the Greek word for inextinguishable, asbestos is widely used in the manufacturing process for a multitude of products and therefore is prevalent in a significant portion of the world's communities, particularly in developing nations (188) making it a relevant human health hazard of the present and future (189).

Exposure to asbestos occurs overwhelmingly through inhalation and leads to a litany of diseases including lung fibrosis (asbestosis), lung carcinoma and malignant mesothelioma (MM) (190). Intriguingly there is an additive risk of lung cancer when cigarette smoking is combined with asbestos exposure (191). Asbestos fibers are known to first interact with

the upper-respiratory tract and exhibit more lasting effects on lung epithelial cells and resident macrophages, with the fiber geometry dictating how deep into the lungs the asbestos travels (longer, thinner fibers are capable of traveling further) (189, 192).

MM is defined as a highly locally-invasive cancer which develops from mesothelial cells that line the body's cavities. Once exposed to asbestos, there is a remarkably long latency period before MM develops, typically around 10-50 years. Furthermore, once MM is diagnosed, it is fatal within 6-12 months (193). As noted the main determinate cause of MM is exposure to asbestos, and unfortunately there are currently no conclusive biomarkers for identifying exposure to asbestos or for early diagnosis of MM. Moreover, therapeutic strategies for MM are lacking as there are no successful regimens for fighting this disease after onset, with chemotherapeutic administration of pemetrexed and cisplatin being the only licensed approach (194). The mechanism by which this cancer develops in the first place, after asbestos exposure, is also less understood and by delineating the molecular pathways involved, we can gain a foothold of understanding that would no doubt lead to improvements in diagnosis and therapy.

As there are clearly large gaps in the knowledge surrounding MM disease development, onset, treatment, and the minimal presence of potential biomarkers for asbestos exposure and early diagnosis, there exists potential to forward our understanding by delving into the realm of exosome research. This review will provide a brief summary of the current literature and experimental knowledge on MM and asbestos exposure as it pertains to advances in exosome-centered investigations.

Malignant mesothelioma and exosomes

The first inquiry into exosomes and MM was a focused effort to identifying exosomes and their protein cargo from human caner pleural effusions. Exosomes were isolated by sucrose-gradient ultracentrifugation from the pleural fluid of patients suffering from MM, lung cancer, breast cancer and ovarian cancer. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analysis indicated large amounts of peptides originating from immunoglobulins and various complement factors, as well as previously undescribed exosomal proteins such as sorting nexing (SNX25) protein, B-cell translocation gene 1 (BTG1) and pigment epithelium-derived factor (PEDF) (195). Both BTG1 and PEDF were in increased abundance in exosomes from malignant processes which may designate as being involved in tumor exosome biogenesis, according to Bard et al. (2004). Moreover, Western blot analysis verified the presence of MHC class II molecule, HSP90, and immunoglobulin G and M.

As indicated in the publication, before their results can be generalized, the risk of contaminating proteins that elute with exosomal proteins in these effusions needs to be taken into account. Although pleural effusions contain exosomes from many cellular origins not limited to tumor cells themselves, this report was an important first step in relating exosomes to MM, cancer and isolation of possible biomarkers from pleural effusions.

As a follow-up to their first study to entrench upon the paradigm of exosome research in MM, the Lambrecht group (196) conducted a descriptive effort on the protein composition of exosomes that are secreted from MM tumor cells. They chose to study MM due to the limited knowledge of tumor antigens in the disease, and employed MALDI-TOF mass

spectrometry to outline the proteomic cargo of MM exosomes. MM tumor cell-lines were created from 10 patients diagnosed with MM, and exosomes were isolated from 7 of these tumor cell lines using ultra-centrifugation and characterized by TEM for their cup-shaped morphology and size range. Exosomal proteins were subjected to MALDI-TOF analysis and of the 38 identified proteins, four were confirmed by Western blot analysis: fascin, β-tubulin, HSC70 and HSP90 (196).

In addition, as reported in in vivo systems (197), these tumor exosomes were also enriched with MHC class I molecules, and the authors also indicated high levels of annexins which may be involved in membrane-cytoskeleton dynamics. This report by Hegmans et al. (2004) revealed several proteins that had not yet been indicated on tumor exosomes or in MM cell lines, therefore providing novel information on MM and tumor exosomes as a whole that could advance our understanding of the disease.

In 2005, Clayton et al. published their work on the immunological functions of exosomes secreted by tumor cells (breast cancer and mesothelioma), and how these tumor exosomes altered the expression of the NKG2D receptor on target blood leukocytes. The exosomes secreted from these MM cancer cells turned out to be positive in expression of NKG2D ligands, and this was directly related the capacity of MM exosomes to decrease the capacity of effector T cells to kill target cells (198).

In the study, it was demonstrated that the two MM cell lines used had high expression of NKG2D ligands (as well as positive staining for MICA, MICB and ULBP-3), and appeared to correlate with the MM exosomes' aptitude in more effectively suppressing NKG2D expression on target cells. Overall, this report indicates a role of MM exosomes in

phenotypically altering immune cells in a way that can aid tumor cells in immune evasion by the presence of exosome ligands to NKG2D.

A promising field of therapeutic cancer research of late is focused on the use of tumor-associated antigens (TAA) present on tumor exosomes as a mode of dendritic cell based immunotherapy. The concept being that tumor exosomes bearing TAAs, mostly secreted from immunogenic cancers, are adept at inducing anti-tumor responses in mouse cancer models by activation of dendritic cells. An intriguing display of this potential was reported by Mahaweni et al.(199), except that by using MM cells, they incorporated a rather unprecedented step forward in this field because MM is regarded as a non-immunogenic cancer with very few TAAs known. Their investigation assessed if MM exosomes were potential antigen sources for dendritic cell based immunotherapy (199).

Initially, a lethal dose of MM tumor cells were injected into BALB/c mice. After seven days of tumor formation in the mice, a single bolus dose of dendritic cells were injected into the tumor-bearing mice for immunotherapy. These dendritic cells, however, had been loaded with either MM exosomes or MM cell lysate (or PBS control) to quarry if the exosomes had an immunogenically priming capacity on the dendritic cells. The overall median survival of tumor bearing mice was significantly increased in the dendritic cell immunotherapy loaded with MM tumor exosomes compared to cell lysate indicating that there may be some promise in using MM exosomes as immunotherapy, as well as in other non-immunogenic tumors.

The subsequent research regarding exosomes in MM had an intriguing focus on the formation of tunneling nanotubes (TnTs), the actin–based cellular extensions involved in

intercellular cargo transport. The relationship between TnT formation and their communicatory effects with MM tumorigenesis is unknown, and for their study, Thayanithy et al.(200) centered in on exosomes as possible mediators for TnT formation in MM. MM exosomes were purified and added to dishes of independently cultured MM cells, and it was found that in these conditions, MM tumor cells produced significantly more TnTs than cells cultured without exogenous exosome addition (200).

The researchers indicated that the added tumor exosomes enriched at the base of, and inside the TnTs, which correlates interestingly to a 2016 report (201) on the mode of exosomal interaction with target cells. In the study by Heusermann et al., exosomes were demonstrated to localize and "surf" on filipodia (similar actin filamentous cellular projections) before internalization (201, 202). The uptake of MM exosomes by MM cells apparently facilitated more TnT connections between tumor cells, and connected cells had nearly twice the number of lipid-raft enriched regions. Taken together, it can be seen that MM exosomes may act as an induction agent of TnT formation between MM tumor cells, and perhaps this connection is an important conduit of cellular information vital to MM progression.

Progressing on the understanding of the MM secretome, Greening et al. (203) released a comprehensive study on MM derived exosomal proteomic cargo. By use of quantitative proteomics, they delineated the protein make up of exosomes from 4 human MM cell lines and identified a total 2,178 proteins from all cells, with 631 common exosomal proteins between all groups (203). As this report came after the aforementioned exosomal inquiries in MM, there were several common proteins identified in the previous report (195),

however, 2,073 proteins were unique to this investigation. Of their MM exosome proteins, the investigators demarcated candidate biomarkers based on clinical relevance, amongst them: tubulin isotypes TUBB4A, Q8IWP6, B3KPS3; galectin-3-binding protein and LGB3P; alpha enolase, annexin 1 and G6PD. Furthermore, it was identified that MM exosomes contained mesothelin, calreticulin, vimentin, and superoxide dismutase, all known to be expressed highly in MM. Additionally, the results of this research uncovered the presence of 26 immunoregulatory components in MM exosomes (such as oncostatin-M receptor (OSMR), multidrug resistance-associated protein 1 (ABCC1), and the SUMO-1 activating receptor, SAE1), as well as 16 tumor-derived antigens, including glypican-1, which has been identified in many tumor derived exosomes and is recorded as potentially valuable biomarker for pancreatic cancer (204). Importantly, this study also provided valuable insight that showed that MM exosomes regulate the cells of the tumor microenvironment by increasing the migratory capacity of fibroblasts and endothelial cells in vitro. Together, their findings implicate MM exosomes as containing many proteins relevant to cancer, angiogenesis, metastasis, migration and immune regulation.

The Robinson group provided another iteration on their quests for elucidating the complexities of the MM secretome suing iTRAQ proteomic analysis. Using 6 MM cell lines in comparison to 3 primary mesothelial cell cultures, it was seen that MM cell secretomes contained higher abundances of exosomal proteins (205). This study is primarily focused on the whole secretome with only some references to exosomes.

The literature review presented above is 100% focused on exosomal content/signature from MM cells and how exosomes can help in communication between MM cells. However, the

role exosomes can play in development of MM or help in early diagnosis of MM is missing. For more than a decade our lab has been interested in uncovering mechanisms of MM development in response to asbestos exposure. Based on the fact that asbestos is inhaled into the lung, yet MM develops in remotely present pleural and peritoneal mesothelial cells, we were encouraged to focus on exosomes as a carrier of information from lung cells to mesothelial cells. As a first of its kind study our lab investigated the proteomic cargo and gene modulatory effects of exosomes from asbestos-exposed cells. Our investigation began by culturing lung epithelial cells (BEAS2B) or macrophages (THP1) (the first known cells to encounter asbestos upon inhalation) with asbestos and isolating their exosomes. These asbestos-exosomes were subjected to tandem-mass spectrometry for protein identification. It was shown that 145 proteins were identified in epithelial cell exosomes and 55 were significantly different in abundance in the asbestos exposed group including plasminogen activator inhibitor 1, vimentin, thrombospondin and glypican-1 (206). We next assessed that the exosomes from asbestos exposed epithelial cells led to genetic changes in target primary pleural human mesothelial cells (HPM3) that were reminiscent of epithelial to mesenchymal transition (EMT): down-regulation of E-cadherin, desmoplakin and IL1 receptor antagonist (206).

Upon proteomic analysis of macrophage exosomes, we (Munson et al. 2018) identified 785 proteins. Of these proteins, 32 had significantly different abundances between exosomes from the asbestos exposed group and the control. Fifteen of these exosomal proteins were in greater abundance in the asbestos group compared to control and interestingly, vimentin and SOD were amongst those that increase in exosomes from macrophages after asbestos

exposure. In response to exposure of asbestos exosomes from macrophages to target primary mesothelial cells, it was shown that significant genetic alterations occur in mesothelial cells: 498 gene changes total (with 1.5 fold cutoff with an ANOVA transcript level p-value less than 0.05), 241 up and 257 down-regulated. As a positive control, the group used asbestos fibers on mesothelial cells, and uncovered that 206 genes were mutually altered in the asbestos-exosomes exposed, or asbestos exposed group of mesothelial cells. Three up- (*hCCNB2*, *hEGR1* and *hFANCD2*) and down-regulated (*hCRELD2*, *hERO1B* and *hJAG1*) genes were then validated by qPCR (206). Of note is that CCNB2 overexpression is attributed with MM and FANCD2 is up-regulated in MM and asbestos exposure (207, 208). This exciting discovery, is novel in that it implicates exosomes from asbestos exposed cells as being capable of changing mesothelial cell genetics in ways similar to how asbestos fibers would on their own. As a next step, this information will be verified in *in vivo* systems for future studies.

As an initial step in the direction of *in vivo* study we committed our efforts to defining the proteomic signature of mouse serum exosomes in an asbestos exposure model. C57/Bl6 mice were exposed to asbestos via oropharyngeal aspiration, and 56 days later, serum exosomes were isolated for proteomic analysis. Again using tandem-mass spectrometry for protein identification, we showed that there were 376 quantifiable proteins in the mouse serum exosomes, with the majority of protein being more abundant in the asbestos exposed group (209). Of these more abundant proteins in the asbestos exposed group, three were validated by Western blot analysis, all of which are acute-phase proteins: haptoglobin; ceruloplasmin, the copper carrying glycoprotein previously seen to be increased in MM

patients' blood and asbestos exposed individuals (210); and fibulin-1, a member of the fibulin family, of which, fibulin-3 has been suggested as being implicative of asbestos exposure and MM (211). We did not see common exosomal proteins between our 2 published studies as these are very different systems, *in vitro* vs *in vivo* and human vs mouse.

In addition to the above mentioned published studies, we have also performed numerous studies with human mesothelioma cells, plasma from asbestos exposed and mesothelioma patient samples. We do find some common signatures between our study and others, including SOD, vimentin, and glypican-1 (203, 205). Studies were also performed with plasma exosomes isolated from healthy volunteers, asbestos exposed non-tumor group and asbestos exposed mesothelioma groups. Although the number of exosomes per ml of plasma were not different in various groups, the exosomal protein quantity was more in different disease groups as compared to controls. Proteomic analysis performed on these samples showed the presence of coagulation-related proteins in exosomes from the disease group (mesothelioma and asbestos-exposed) as compared to control. Control group plasma exosomes presented a signature including immunoglobulins, lipoproteins and platelet-related proteins. These data indicate altered immune surveillance in MM samples concomitant with the increase of coagulation factors (unpublished data). We plan to repeat/validate these studies with larger sample size before publishing the data.

Conclusion

Asbestos exposure is a serious health concern for thousands of people worldwide, and MM is the cancer resulting primarily from asbestos exposure. To date, there are no successful therapeutic regimens for treating MM, and with the dismal survival time after diagnosis and lack of biomarkers for early detection make it an important area for propagating research. The field of exosomes in cancer has exploded recently due to the fact that these extracellular vesicles are emerging players in the dynamics of cancer biology, contributing to cellular crosstalk involved in cancerous processes and housing cancer biomarker signatures. Many advances have been made to date using exosomes for biomarker identification, detecting novel therapeutic targets, and basic understanding of tumor biology (212, 213). In this regard, using exosomes to gain needed insight into MM development, detecting potential biomarkers, pinpointing therapeutic targets and harnessing exosomes as drug delivery devices and immunoregulators against cancer are the next big steps researchers must take.

The studies reviewed above provide the initial framework for understanding possible biomarkers and the underlying biology of MM and asbestos exposure. From their findings, research can commit to further identifying means of early detection of asbestos exposure or asbestos-related disease development, as well as uncovering much needed therapeutic targets. Moreover, the ability to understand the mechanistics of MM development and progression in regards to exosomes is an important realm that may be utilized in treating MM cancer patients. Ultimately, we hope that exosome research in MM continues on this forward trajectory and more significant findings are made into the figuring out how

asbestos causes cancer and finding ways to identify dangerous exposure to asbestos and early cancer detection before a fatal diagnosis is made.

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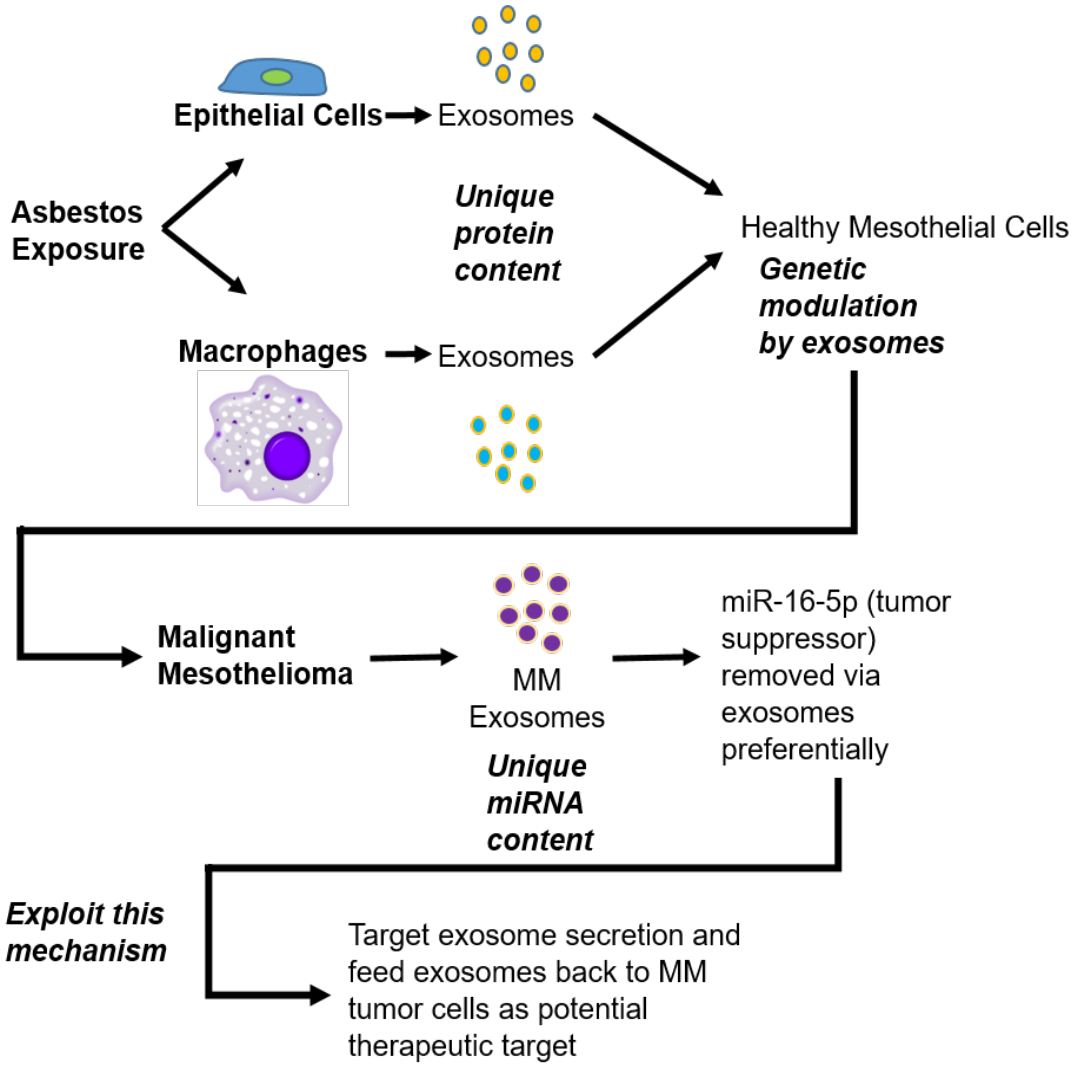

Figure 6. Schematic overview of themes discussed in this dissertation outlining the chronological reasoning of experiments and broad concepts. The following chapters delve into the experiments and characterizations that led to the reasoning that asbestos exposure of lung epithelial cell and macrophages leads to exosomes with unique protein cargo that travels to target mesothelial cells and alters them genetically. This alteration leads to potential development of malignant mesothelioma (MM) and MM tumor cells secrete exosomes with unique miRNA cargo. These MM exosomes turned out to be a

route of removing tumor suppressor miRNAs, namely, miR-16-5p. We went to exploit this process by inhibiting exosome secretion and force-feeding MM tumor exosomes back to MM cells as a potential advance in therapeutic targeting.

References

1. Zukowski R, Gaze R. Tensile Strength of Asbestos. Nature. 1959;183(4653):35-7.
2. Wagner JC, Sleggs CA, Marchand P. Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. British journal of industrial medicine. 1960;17:260-71.
3. Mossman BT, Gee JB. Asbestos-related diseases. N Engl J Med. 1989;320(26):1721-30.
4. Mossman BT. Carcinogenesis and related cell and tissue responses to asbestos: a review. The Annals of occupational hygiene. 1994;38(4):617-24, 423.
5. Currie GP, Watt SJ, Maskell NA. An overview of how asbestos exposure affects the lung. BMJ. 2009;339:b3209.
6. Mossman BT, Marsh JP. Evidence supporting a role for active oxygen species in asbestos-induced toxicity and lung disease. Environmental health perspectives. 1989;81:91-4.
7. Lund LG, Aust AE. Iron-catalyzed reactions may be responsible for the biochemical and biological effects of asbestos. BioFactors (Oxford, England). 1991;3(2):83-9.
8. Kamp DW, Graceffa P, Pryor WA, Weitzman SA. The role of free radicals in asbestos-induced diseases. Free radical biology & medicine. 1992;12(4):293-315.
9. Lazarus AA, Philip A. Asbestosis. Disease-a-month : DM. 2011;57(1):14-26.
10. Hillerdal G. Asbestos related pleuropulmonary lesions and the erythrocyte sedimentation rate. Thorax. 1984;39(10):752-8.
11. Metintas M, Metintas S, Hillerdal G, Ucgun I, Erginel S, Alatas F, et al. Nonmalignant pleural lesions due to environmental exposure to asbestos: a field-based, cross-sectional study. European Respiratory Journal. 2005;26(5):875-80.
12. Manning CB, Vallyathan V, Mossman BT. Diseases caused by asbestos: mechanisms of injury and disease development. International immunopharmacology. 2002;2(2-3):191-200.
13. Craighead JE, Mossman BT. The pathogenesis of asbestos-associated diseases. N Engl J Med. 1982;306(24):1446-55.
14. Norbet C, Joseph A, Rossi SS, Bhalla S, Gutierrez FR. Asbestos-related lung disease: a pictorial review. Current problems in diagnostic radiology. 2015;44(4):371-82.
15. Craighead JE, Mossman BT. The Pathogenesis of Asbestos-Associated Diseases. New England Journal of Medicine. 1982;306(24):1446-55.
16. Sekido Y. Molecular pathogenesis of malignant mesothelioma. Carcinogenesis. 2013;34(7):1413-9.
17. Beckett P, Edwards J, Fennell D, Hubbard R, Woolhouse I, Peake MD. Demographics, management and survival of patients with malignant pleural mesothelioma in the National Lung Cancer Audit in England and Wales. Lung cancer (Amsterdam, Netherlands). 2015;88(3):344-8.
18. Lee YC, Light RW, Musk AW. Management of malignant pleural mesothelioma: a critical review. Current opinion in pulmonary medicine. 2000;6(4):267-74.
19. Carbone M, Pass HI, Rizzo P, Marinetti M, Di Muzio M, Mew DJ, et al. Simian virus 40-like DNA sequences in human pleural mesothelioma. Oncogene. 1994;9(6):1781-90.
20. Miserocchi G, Sancini G, Mantegazza F, Chiappino G. Translocation pathways for inhaled asbestos fibers. Environmental health : a global access science source. 2008;7:4-.
21. Robinson BW, Lake RA. Advances in malignant mesothelioma. N Engl J Med. 2005;353(15):1591-603.
22. Shukla A, Hillegass JM, MacPherson MB, Beuschel SL, Vacek PM, Butnor KJ, et al. ERK2 is essential for the growth of human epithelioid malignant mesotheliomas. International journal of cancer. 2011;129(5):1075-86.

23. Thompson JK, Shukla A, Leggett AL, Munson PB, Miller JM, MacPherson MB, et al. Extracellular signal regulated kinase 5 and inflammasome in progression of mesothelioma. Oncotarget. 2018;9(1):293-305.
24. Davidson B. Expression of cancer-associated molecules in malignant mesothelioma. Biomarker insights. 2007;2:173-84.
25. Tranchant R, Quetel L, Tallet A, Meiller C, Renier A, de Koning L, et al. Co-occurring Mutations of Tumor Suppressor Genes, LATS2 and NF2, in Malignant Pleural Mesothelioma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2017;23(12):3191-202.
26. Melaiu O, Gemignani F, Landi S. The genetic susceptibility in the development of malignant pleural mesothelioma. Journal of thoracic disease. 2018;10(Suppl 2):S246-S52.
27. Hylebos M, Van Camp G, van Meerbeeck JP, Op de Beeck K. The Genetic Landscape of Malignant Pleural Mesothelioma: Results from Massively Parallel Sequencing. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer. 2016;11(10):1615-26.
28. Welker L, Muller M, Holz O, Vollmer E, Magnussen H, Jorres RA. Cytological diagnosis of malignant mesothelioma--improvement by additional analysis of hyaluronic acid in pleural effusions. Virchows Archiv : an international journal of pathology. 2007;450(4):455-61.
29. Husain AN, Colby TV, Ordonez NG, Allen TC, Attanoos RL, Beasley MB, et al. Guidelines for Pathologic Diagnosis of Malignant Mesothelioma 2017 Update of the Consensus Statement From the International Mesothelioma Interest Group. Archives of pathology & laboratory medicine. 2018;142(1):89-108.
30. Linch M, Gennatas S, Kazikin S, Iqbal J, Gunapala R, Priest K, et al. A serum mesothelin level is a prognostic indicator for patients with malignant mesothelioma in routine clinical practice. BMC cancer. 2014;14:674-.
31. Pass HI, Lott D, Lonardo F, Harbut M, Liu Z, Tang N, et al. Asbestos exposure, pleural mesothelioma, and serum osteopontin levels. N Engl J Med. 2005;353(15):1564-73.
32. Pass HI, Levin SM, Harbut MR, Melamed J, Chiriboga L, Donington J, et al. Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. N Engl J Med. 2012;367(15):1417-27.
33. Creaney J, Dick IM, Meniawy TM, Leong SL, Leon JS, Demelker Y, et al. Comparison of fibulin-3 and mesothelin as markers in malignant mesothelioma. Thorax. 2014;69(10):895-902.
34. Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Ruffie P, et al. Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2003;21(14):2636-44.
35. Bibby AC, Tsim S, Kanellakis N, Ball H, Talbot DC, Blyth KG, et al. Malignant pleural mesothelioma: an update on investigation, diagnosis and treatment. European respiratory review : an official journal of the European Respiratory Society. 2016;25(142):472-86.
36. MacLeod N, Chalmers A, O'Rourke N, Moore K, Sheridan J, McMahon L, et al. Is Radiotherapy Useful for Treating Pain in Mesothelioma?: A Phase II Trial. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer. 2015;10(6):944-50.
37. Flores RM. The mesothelioma surgery shift. The Journal of thoracic and cardiovascular surgery. 2016;151(2):485-6.
38. Flores RM. Pleurectomy decortication for mesothelioma: The procedure of choice when possible. The Journal of thoracic and cardiovascular surgery. 2016;151(2):310-2.

39. Dozier J, Zheng H, Adusumilli PS. Immunotherapy for malignant pleural mesothelioma: current status and future directions. Transl Lung Cancer Res. 2017;6(3):315-24.
40. Hassan R, Bullock S, Premkumar A, Kreitman RJ, Kindler H, Willingham MC, et al. Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin given as a bolus I.V. infusion to patients with mesothelin-expressing mesothelioma, ovarian, and pancreatic cancers. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(17):5144-9.
41. Hassan R, Sharon E, Thomas A, Zhang J, Ling A, Miettinen M, et al. Phase 1 study of the antimesothelin immunotoxin SS1P in combination with pemetrexed and cisplatin for front-line therapy of pleural mesothelioma and correlation of tumor response with serum mesothelin, megakaryocyte potentiating factor, and cancer antigen 125. Cancer. 2014;120(21):3311-9.
42. Bakker E, Guazzelli A, Ashtiani F, Demonacos C, Krstic-Demonacos M, Mutti L. Immunotherapy advances for mesothelioma treatment. Expert review of anticancer therapy. 2017;17(9):799-814.
43. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. Science (New York, NY). 2015;348(6230):62-8.
44. Adusumilli PS, Cherkassky L, Villena-Vargas J, Colovos C, Servais E, Plotkin J, et al. Regional delivery of mesothelin-targeted CAR T cell therapy generates potent and long-lasting CD4-dependent tumor immunity. Science translational medicine. 2014;6(261):261ra151.
45. De Santi C, Melaiu O, Bonotti A, Cascione L, Di Leva G, Foddis R, et al. Deregulation of miRNAs in malignant pleural mesothelioma is associated with prognosis and suggests an alteration of cell metabolism. Scientific reports. 2017;7(1):3140.
46. Gee GV, Koestler DC, Christensen BC, Sugarbaker DJ, Ugolini D, Ivaldi GP, et al. Downregulated microRNAs in the differential diagnosis of malignant pleural mesothelioma. International journal of cancer. 2010;127(12):2859-69.
47. Micolucci L, Akhtar MM, Olivieri F, Rippo MR, Procopio AD. Diagnostic value of microRNAs in asbestos exposure and malignant mesothelioma: systematic review and qualitative meta-analysis. Oncotarget. 2016;7(36):58606-37.
48. Reid G, Pel ME, Kirschner MB, Cheng YY, Mugridge N, Weiss J, et al. Restoring expression of miR-16: a novel approach to therapy for malignant pleural mesothelioma. Annals of oncology : official journal of the European Society for Medical Oncology. 2013;24(12):3128-35.
49. Trams EG, Lauter CJ, Salem N, Jr., Heine U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. Biochimica et biophysica acta. 1981;645(1):63-70.
50. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. Immunology letters. 2006;107(2):102-8.
51. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. The Journal of cell biology. 2013;200(4):373-83.
52. Kahlert C, Melo SA, Protopopov A, Tang J, Seth S, Koch M, et al. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. J Biol Chem. 2014;289(7):3869-75.
53. Lo Cicero A, Stahl PD, Raposo G. Extracellular vesicles shuffling intercellular messages: for good or for bad. Current opinion in cell biology. 2015;35:69-77.
54. Lee Y, El Andaloussi S, Wood MJ. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. Hum Mol Genet. 2012;21(R1):R125-34.
55. Andaloussi EL, Mager I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. Nature reviews Drug discovery. 2013;12(5):347-57.

56. Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. Journal of cell science. 2013;126(Pt 24):5553-65.
57. Friand V, David G, Zimmermann P. Syntenin and syndecan in the biogenesis of exosomes. Biology of the cell / under the auspices of the European Cell Biology Organization. 2015.
58. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annual review of cell and developmental biology. 2014;30:255-89.
59. Kreimer S, Belov AM, Ghiran I, Murthy SK, Frank DA, Ivanov AR. Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics. Journal of proteome research. 2015;14(6):2367-84.
60. Irion U, St Johnston D. bicoid RNA localization requires specific binding of an endosomal sorting complex. Nature. 2007;445(7127):554-8.
61. Ludwig AK, Giebel B. Exosomes: small vesicles participating in intercellular communication. The international journal of biochemistry & cell biology. 2012;44(1):11-5.
62. Buschow SI, van Balkom BW, Aalberts M, Heck AJ, Wauben M, Stoorvogel W. MHC class II-associated proteins in B-cell exosomes and potential functional implications for exosome biogenesis. Immunology and cell biology. 2010;88(8):851-6.
63. de Gassart A, Geminard C, Fevrier B, Raposo G, Vidal M. Lipid raft-associated protein sorting in exosomes. Blood. 2003;102(13):4336-44.
64. Janas T, Janas MM, Sapon K, Janas T. Mechanisms of RNA loading into exosomes. FEBS letters. 2015;589(13):1391-8.
65. Chen K, Rajewsky N. The evolution of gene regulation by transcription factors and microRNAs. Nature reviews Genetics. 2007;8(2):93-103.
66. Bobrie A, Colombo M, Raposo G, Thery C. Exosome secretion: molecular mechanisms and roles in immune responses. Traffic (Copenhagen, Denmark). 2011;12(12):1659-68.
67. Gross JC, Chaudhary V, Bartscherer K, Boutros M. Active Wnt proteins are secreted on exosomes. Nature cell biology. 2012;14(10):1036-45.
68. Wubbolts R, Leckie RS, Veenhuizen PT, Schwarzmann G, Mobius W, Hoernschemeyer J, et al. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. J Biol Chem. 2003;278(13):10963-72.
69. Rana S, Yue S, Stadel D, Zoller M. Toward tailored exosomes: the exosomal tetraspanin web contributes to target cell selection. The international journal of biochemistry & cell biology. 2012;44(9):1574-84.
70. Brinton LT, Sloane HS, Kester M, Kelly KA. Formation and role of exosomes in cancer. Cellular and molecular life sciences : CMLS. 2015;72(4):659-71.
71. Ginestra A, La Placa MD, Saladino F, Cassarà D, Nagase H, Vittorelli ML. The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. Anticancer Res. 1998;18(5A):3433-7.
72. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nature reviews Cancer. 2009;9(4):239-52.
73. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nature medicine. 2013;19(11):1423-37.

74. Webber J, Steadman R, Mason MD, Tabi Z, Clayton A. Cancer exosomes trigger fibroblast to myofibroblast differentiation. Cancer research. 2010;70(23):9621-30.
75. Millimaggi D, Mari M, D'Ascenzo S, Carosa E, Jannini EA, Zucker S, et al. Tumor vesicle-associated CD147 modulates the angiogenic capability of endothelial cells. Neoplasia (New York, NY). 2007;9(4):349-57.
76. Vella LJ. The emerging role of exosomes in epithelial-mesenchymal-transition in cancer. Frontiers in oncology. 2014;4:361.
77. Greening DW, Gopal SK, Mathias RA, Liu L, Sheng J, Zhu HJ, et al. Emerging roles of exosomes during epithelial-mesenchymal transition and cancer progression. Seminars in cell & developmental biology. 2015;40:60-71.
78. Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nature cell biology. 2008;10(5):619-24.
79. Sung BH, Ketova T, Hoshino D, Zijlstra A. Directional cell movement through tissues is controlled by exosome secretion. 2015;6:7164.
80. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nature medicine. 2012;18(6):883-91.
81. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nature cell biology. 2015;17(6):816-26.
82. Zomer A, Maynard C, Verweij FJ, Kamermans A, Schafer R, Beerling E, et al. In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. Cell. 2015;161(5):1046-57.
83. Neviani P, Fabbri M. Exosomic microRNAs in the Tumor Microenvironment. Frontiers in medicine. 2015;2:47.
84. Melo SA, Sugimoto H, O'Connell JT, Kato N, Villanueva A, Vidal A, et al. Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. Cancer cell. 2014;26(5):707-21.
85. Le MTN, Hamar P, Guo C, Basar E, Perdig, xE, et al. miR-200–containing extracellular vesicles promote breast cancer cell metastasis. The Journal of Clinical Investigation. 2014;124(12):5109-28.
86. Valenzuela MM, Ferguson Bennit HR, Gonda A, Diaz Osterman CJ, Hibma A, Khan S, et al. Exosomes Secreted from Human Cancer Cell Lines Contain Inhibitors of Apoptosis (IAP). Cancer microenvironment : official journal of the International Cancer Microenvironment Society. 2015.
87. Peng P, Yan Y, Keng S. Exosomes in the ascites of ovarian cancer patients: origin and effects on anti-tumor immunity. Oncology reports. 2011;25(3):749-62.
88. Miller IV, Grunewald TG. Tumour-derived exosomes: Tiny envelopes for big stories. Biology of the cell / under the auspices of the European Cell Biology Organization. 2015.
89. Szczepanski MJ, Szajnik M, Welsh A, Whiteside TL, Boyiadzis M. Blast-derived microvesicles in sera from patients with acute myeloid leukemia suppress natural killer cell function via membrane-associated transforming growth factor-beta1. Haematologica. 2011;96(9):1302-9.
90. Chaput N, Thery C. Exosomes: immune properties and potential clinical implementations. Seminars in immunopathology. 2011;33(5):419-40.

91. Chen WX, Liu XM, Lv MM, Chen L, Zhao JH, Zhong SL, et al. Exosomes from drug-resistant breast cancer cells transmit chemoresistance by a horizontal transfer of microRNAs. PLoS One. 2014;9(4):e95240.
92. Zhao L, Liu W, Xiao J, Cao B. The role of exosomes and "exosomal shuttle microRNA" in tumorigenesis and drug resistance. Cancer letters. 2015;356(2 Pt B):339-46.
93. Ji R, Zhang B, Zhang X, Xue J, Yuan X, Yan Y, et al. Exosomes derived from human mesenchymal stem cells confer drug resistance in gastric cancer. Cell cycle (Georgetown, Tex). 2015:0.
94. Braicu C, Tomuleasa C, Monroig P, Cucuianu A, Berindan-Neagoe I, Calin GA. Exosomes as divine messengers: are they the Hermes of modern molecular oncology? Cell death and differentiation. 2015;22(1):34-45.
95. Vicencio JM, Yellon DM, Sivaraman V, Das D, Boi-Doku C, Arjun S, et al. Plasma exosomes protect the myocardium from ischemia-reperfusion injury. Journal of the American College of Cardiology. 2015;65(15):1525-36.
96. Sahoo S, Losordo DW. Exosomes and cardiac repair after myocardial infarction. Circulation research. 2014;114(2):333-44.
97. Kumar S. Bone defect repair in mice by mesenchymal stem cells. Methods in molecular biology (Clifton, NJ). 2014;1213:193-207.
98. Arntz OJ, Pieters BC, Oliveira MC, Broeren MG, Bennink MB, de Vries M, et al. Oral administration of bovine milk-derived extracellular vesicles attenuates arthritis in two mouse models. Molecular nutrition & food research. 2015.
99. Ching RC, Kingham PJ. The role of exosomes in peripheral nerve regeneration. Neural regeneration research. 2015;10(5):743-7.
100. Saenz-Cuesta M, Osorio-Querejeta I, Otaegui D. Extracellular Vesicles in Multiple Sclerosis: What are They Telling Us? Frontiers in cellular neuroscience. 2014;8:100.
101. Haney MJ, Klyachko NL, Zhao Y, Gupta R, Plotnikova EG, He Z, et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. Journal of controlled release : official journal of the Controlled Release Society. 2015;207:18-30.
102. Zarovni N, Corrado A, Guazzi P, Zocco D, Lari E, Radano G, et al. Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches. Methods. 2015.
103. Lazar I, Clement E, Ducoux-Petit M, Denat L, Soldan V, Dauvillier S, et al. Proteome characterization of melanoma exosomes reveals a specific signature for metastatic cell lines. Pigment cell & melanoma research. 2015;28(4):464-75.
104. Alegre E, Sammamed M, Fernandez-Landazuri S, Zubiri L, Gonzalez A. Circulating biomarkers in malignant melanoma. Advances in clinical chemistry. 2015;69:47-89.
105. Chiam K, Wang T, Watson DI, Mayne GC, Irvine TS, Bright T, et al. Circulating Serum Exosomal miRNAs As Potential Biomarkers for Esophageal Adenocarcinoma. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2015;19(7):1208-15.
106. Davalieva K, Polenakovic M. Proteomics in diagnosis of prostate cancer. Prilozi / Makedonska akademija na naukite i umetnostite, Oddelenie za bioloski i medicinski nauki = Contributions / Macedonian Academy of Sciences and Arts, Section of Biological and Medical Sciences. 2015;36(1):5-36.
107. Mahmoudi K, Ezrin A, Hadjipanayis C. Small extracellular vesicles as tumor biomarkers for glioblastoma. Molecular aspects of medicine. 2015.

108. Hornick NI, Huan J, Doron B, Goloviznina NA, Lapidus J, Chang BH, et al. Serum Exosome MicroRNA as a Minimally-Invasive Early Biomarker of AML. Sci Rep. 2015;5:11295.
109. Cha DJ, Franklin JL, Dou Y, Liu Q, Higginbotham JN, Demory Beckler M, et al. KRAS-dependent sorting of miRNA to exosomes. eLife. 2015;4.
110. Matsumura T, Sugimachi K, Iinuma H, Takahashi Y, Kurashige J, Sawada G, et al. Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. Br J Cancer. 2015.
111. Zhou X, Zhu W, Li H, Wen W, Cheng W, Wang F, et al. Diagnostic value of a plasma microRNA signature in gastric cancer: a microRNA expression analysis. Sci Rep. 2015;5:11251.
112. Zhou J, Gong G, Tan H, Dai F, Zhu X, Chen Y, et al. Urinary microRNA-30a-5p is a potential biomarker for ovarian serous adenocarcinoma. Oncology reports. 2015;33(6):2915-23.
113. Tang MK, Wong AS. Exosomes: Emerging biomarkers and targets for ovarian cancer. Cancer letters. 2015.
114. Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nature. 2015.
115. Dutta S, Reamtong O, Pangvongsa W, Kitdumrongthum S, Janpipatkul K, Sangvanich P, et al. Proteomics profiling of cholangiocarcinoma exosomes: A potential role of oncogenic protein transferring in cancer progression. Biochimica et biophysica acta. 2015.
116. Gumireddy K, Li A, Chang DH, Liu Q, Kossenkov AV, Yan J, et al. AKAP4 is a circulating biomarker for non-small cell lung cancer. Oncotarget. 2015.
117. Muller L, Muller-Haegele S, Mitsuhashi M, Gooding W, Okada H, Whiteside TL. Exosomes isolated from plasma of glioma patients enrolled in a vaccination trial reflect antitumor immune activity and might predict survival. Oncoimmunology. 2015;4(6):e1008347.
118. Sivadasan P, Gupta MK, Sathe GJ, Balakrishnan L, Palit P, Gowda H, et al. Human salivary proteome - a resource of potential biomarkers for oral cancer. Journal of proteomics. 2015.
119. Li Y, Zheng Q, Bao C, Li S, Guo W, Zhao J, et al. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. Cell research. 2015.
120. Chalmin F, Ladoire S, Mignot G, Vincent J, Bruchard M, Remy-Martin JP, et al. Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. J Clin Invest. 2010;120(2):457-71.
121. Phuyal S, Hessvik NP, Skotland T, Sandvig K, Llorente A. Regulation of exosome release by glycosphingolipids and flotillins. The FEBS journal. 2014;281(9):2214-27.
122. Sioud M. RNA interference: mechanisms, technical challenges, and therapeutic opportunities. Methods in molecular biology (Clifton, NJ). 2015;1218:1-15.
123. Vader P, Breakefield XO, Wood MJ. Extracellular vesicles: emerging targets for cancer therapy. Trends in molecular medicine. 2014;20(7):385-93.
124. Bobrie A, Krumeich S, Reyal F, Recchi C, Moita LF, Seabra MC, et al. Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression. Cancer research. 2012;72(19):4920-30.
125. Lima LG, Chammas R, Monteiro RQ, Moreira ME, Barcinski MA. Tumor-derived microvesicles modulate the establishment of metastatic melanoma in a phosphatidylserine-dependent manner. Cancer letters. 2009;283(2):168-75.
126. Johnsen KB, Gudbergsson JM, Skov MN, Pilgaard L, Moos T, Duroux M. A comprehensive overview of exosomes as drug delivery vehicles - endogenous nanocarriers for targeted cancer therapy. Biochimica et biophysica acta. 2014;1846(1):75-87.

127. Katakowski M, Buller B, Zheng X, Lu Y, Rogers T, Osobamiro O, et al. Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. Cancer letters. 2013;335(1):201-4.
128. Gyorgy B, Hung ME, Breakefield XO, Leonard JN. Therapeutic applications of extracellular vesicles: clinical promise and open questions. Annual review of pharmacology and toxicology. 2015;55:439-64.
129. Koppers-Lalic D, Hogenboom MM, Middeldorp JM, Pegtel DM. Virus-modified exosomes for targeted RNA delivery; A new approach in nanomedicine. Advanced Drug Delivery Reviews. 2013;65(3):348-56.
130. Tian Y, Li S, Song J, Ji T, Zhu M, Anderson GJ, et al. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. Biomaterials. 2014;35(7):2383-90.
131. Yang T, Martin P, Fogarty B, Brown A, Schurman K, Phipps R, et al. Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in danio rerio. Pharmaceutical research. 2015;32(6):2003-14.
132. Yin W, Ouyang S, Li Y, Xiao B, Yang H. Immature dendritic cell-derived exosomes: a promise subcellular vaccine for autoimmunity. Inflammation. 2013;36(1):232-40.
133. Hung ME, Leonard JN. Stabilization of exosome-targeting peptides via engineered glycosylation. J Biol Chem. 2015;290(13):8166-72.
134. Lai CP, Mardini O, Ericsson M, Prabhakar S, Maguire CA, Chen JW, et al. Dynamic biodistribution of extracellular vesicles in vivo using a multimodal imaging reporter. ACS nano. 2014;8(1):483-94.
135. Lai CP, Tannous BA, Breakefield XO. Noninvasive in vivo monitoring of extracellular vesicles. Methods in molecular biology (Clifton, NJ). 2014;1098:249-58.
136. Ridder K, Sevko A, Heide J, Dams M, Rupp AK, Macas J, et al. Extracellular vesicle-mediated transfer of functional RNA in the tumor microenvironment. Oncoimmunology. 2015;4(6):e1008371.
137. Yeo RW, Lai RC, Zhang B, Tan SS, Yin Y, Teh BJ, et al. Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. Adv Drug Deliv Rev. 2013;65(3):336-41.
138. Lee JK, Park SR, Jung BK, Jeon YK, Lee YS, Kim MK, et al. Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. PLoS One. 2013;8(12):e84256.
139. Gehrmann U, Hiltbrunner S, Georgoudaki AM, Karlsson MC, Naslund TI, Gabrielsson S. Synergistic induction of adaptive antitumor immunity by codelivery of antigen with alpha-galactosylceramide on exosomes. Cancer research. 2013;73(13):3865-76.
140. Lee EY, Park KS, Yoon YJ, Lee J, Moon HG, Jang SC, et al. Therapeutic effects of autologous tumor-derived nanovesicles on melanoma growth and metastasis. PLoS One. 2012;7(3):e33330.
141. Chargaff E, West R. The biological significance of the thromboplastic protein of blood. J Biol Chem. 1946;166(1):189-97.
142. Wolf P. The Nature and Significance of Platelet Products in Human Plasma. British Journal of Haematology. 1967;13(3):269-88.
143. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. Pharmacological reviews. 2012;64(3):676-705.
144. Piper RC, Katzmann DJ. Biogenesis and Function of Multivesicular Bodies. Annual review of cell and developmental biology. 2007;23:519-47.

145. Blanc L, Vidal M. New insights into the function of Rab GTPases in the context of exosomal secretion. Small GTPases. 2017:1-12.
146. Lane RE, Korbie D, Anderson W, Vaidyanathan R, Trau M. Analysis of exosome purification methods using a model liposome system and tunable-resistive pulse sensing. Sci Rep. 2015;5:7639.
147. Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. Methods in molecular biology (Clifton, NJ). 2015;1295:179-209.
148. Chiou N-T, Ansel KM. Improved exosome isolation by sucrose gradient fractionation of ultracentrifuged crude exosome pellets. 2016.
149. Rood IM, Deegens JK, Merchant ML, Tamboer WP, Wilkey DW, Wetzels JF, et al. Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome. Kidney international. 2010;78(8):810-6.
150. Cheruvanky A, Zhou H, Pisitkun T, Kopp JB, Knepper MA, Yuen PST, et al. Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. American journal of physiology Renal physiology. 2007;292(5):F1657-F61.
151. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. Theranostics. 2017;7(3):789-804.
152. Helwa I, Cai J, Drewry MD, Zimmerman A, Dinkins MB, Khaled ML, et al. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. PLoS One. 2017;12(1):e0170628.
153. Vlassov AV, Magdaleno S, Setterquist R, Conrad R. Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. Biochimica et Biophysica Acta (BBA) - General Subjects. 2012;1820(7):940-8.
154. Wu Y, Deng W, Klinke DJ. Exosomes: Improved methods to characterize their morphology, RNA content, and surface protein biomarkers. The Analyst. 2015;140(19):6631-42.
155. Hoen ENMN-t, van der Vlist EJ, Aalberts M, Mertens HCH, Bosch BJ, Bartelink W, et al. Quantitative and qualitative flow cytometric analysis of nanosized cell-derived membrane vesicles. Nanomedicine: Nanotechnology, Biology and Medicine. 2012;8(5):712-20.
156. Yang F, Liao X, Tian Y, Li G. Exosome separation using microfluidic systems: size-based, immunoaffinity-based and dynamic methodologies. Biotechnology journal. 2017;12(4).
157. Coumans FAW, Brisson AR, Buzas EI, Dignat-George F, Drees EEE, El-Andaloussi S, et al. Methodological Guidelines to Study Extracellular Vesicles. Circulation research. 2017;120(10):1632-48.
158. Skotland T, Sandvig K, Llorente A. Lipids in exosomes: Current knowledge and the way forward. Progress in lipid research. 2017;66:30-41.
159. Vyas N, Dhawan J. Exosomes: mobile platforms for targeted and synergistic signaling across cell boundaries. Cellular and molecular life sciences : CMLS. 2017;74(9):1567-76.
160. Isola AL, Chen S. Exosomes: The Messengers of Health and Disease. Current neuropharmacology. 2017;15(1):157-65.
161. Madison MN, Okeoma CM. Exosomes: Implications in HIV-1 Pathogenesis. Viruses. 2015;7(7):4093-118.
162. Sampaio NG, Cheng L, Eriksson EM. The role of extracellular vesicles in malaria biology and pathogenesis. Malaria journal. 2017;16(1):245.

163. Boulanger CM, Loyer X, Rautou PE, Amabile N. Extracellular vesicles in coronary artery disease. Nature reviews Cardiology. 2017;14(5):259-72.
164. Campion CG, Sanchez-Ferras O, Batchu SN. Potential Role of Serum and Urinary Biomarkers in Diagnosis and Prognosis of Diabetic Nephropathy. Canadian journal of kidney health and disease. 2017;4:2054358117705371.
165. Martinez MC, Andriantsitohaina R. Extracellular Vesicles in Metabolic Syndrome. Circulation research. 2017;120(10):1674-86.
166. Cuffe JSM, Holland O, Salomon C, Rice GE, Perkins AV. Review: Placental derived biomarkers of pregnancy disorders. Placenta. 2017;54:104-10.
167. Wu X, Zheng T, Zhang B. Exosomes in Parkinson's Disease. Neuroscience bulletin. 2017;33(3):331-8.
168. Selmaj I, Mycko MP, Raine CS, Selmaj KW. The role of exosomes in CNS inflammation and their involvement in multiple sclerosis. Journal of neuroimmunology. 2017;306:1-10.
169. Yan S, Han B, Gao S, Wang X, Wang Z, Wang F, et al. Exosome-encapsulated microRNAs as circulating biomarkers for colorectal cancer. Oncotarget. 2017.
170. Takahashi RU, Prieto-Vila M, Hironaka A, Ochiya T. The role of extracellular vesicle microRNAs in cancer biology. Clinical chemistry and laboratory medicine. 2017;55(5):648-56.
171. Rashed MH, Kanlikilicer P, Rodriguez-Aguayo C, Pichler M, Bayraktar R, Bayraktar E, et al. Exosomal miR-940 maintains SRC-mediated oncogenic activity in cancer cells: a possible role for exosomal disposal of tumor suppressor miRNAs. Oncotarget. 2017;8(12):20145-64.
172. Kanlikilicer P, Rashed MH, Bayraktar R, Mitra R, Ivan C, Aslan B, et al. Ubiquitous Release of Exosomal Tumor Suppressor miR-6126 from Ovarian Cancer Cells. Cancer research. 2016;76(24):7194-207.
173. Whiteside TL. Exosomes carrying immunoinhibitory proteins and their role in cancer. Clinical and experimental immunology. 2017.
174. Lobb RJ, van Amerongen R, Wiegmans A, Ham S, Larsen JE, Moller A. Exosomes derived from mesenchymal non-small cell lung cancer cells promote chemoresistance. International journal of cancer. 2017;141(3):614-20.
175. Bach DH, Hong JY, Park HJ, Lee SK. The role of exosomes and miRNAs in drug-resistance of cancer cells. International journal of cancer. 2017;141(2):220-30.
176. Sterzenbach U, Putz U, Low LH, Silke J, Tan SS, Howitt J. Engineered Exosomes as Vehicles for Biologically Active Proteins. Molecular therapy : the journal of the American Society of Gene Therapy. 2017;25(6):1269-78.
177. Luan X, Sansanaphongpricha K, Myers I, Chen H, Yuan H, Sun D. Engineering exosomes as refined biological nanoplatforms for drug delivery. Acta pharmacologica Sinica. 2017;38(6):754-63.
178. Moore C, Kosgodage U, Lange S, Inal JM. The emerging role of exosome and microvesicle- (EMV-) based cancer therapeutics and immunotherapy. International journal of cancer. 2017;141(3):428-36.
179. Syn NL, Wang L, Chow EK, Lim CT, Goh BC. Exosomes in Cancer Nanomedicine and Immunotherapy: Prospects and Challenges. Trends in biotechnology. 2017;35(7):665-76.
180. Shahabipour F, Barati N, Johnston TP, Derosa G, Maffioli P, Sahebkar A. Exosomes: Nanoparticulate tools for RNA interference and drug delivery. Journal of cellular physiology. 2017;232(7):1660-8.
181. Munson P, Shukla A. Exosomes: Potential in Cancer Diagnosis and Therapy. Medicines. 2015;2(4):310-27.

182. Teixeira JH, Silva AM, Almeida MI, Barbosa MA, Santos SG. Circulating extracellular vesicles: Their role in tissue repair and regeneration. Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis. 2016.
183. Smalheiser NR. Exosomal transfer of proteins and RNAs at synapses in the nervous system. Biology direct. 2007;2:35.
184. Ibrahim AG, Cheng K, Marban E. Exosomes as critical agents of cardiac regeneration triggered by cell therapy. Stem cell reports. 2014;2(5):606-19.
185. Madison MN, Okeoma CM. Exosomes: Implications in HIV-1 Pathogenesis. Viruses. 2015;7(7):4093-118.
186. Properzi F, Logozzi M, Fais S. Exosomes: the future of biomarkers in medicine. Biomarkers in medicine. 2013;7(5):769-78.
187. IARC W. Asbestos (chrysotile, amosite, crocidolite, tremolite, actinolite, and anthophyllite). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans A Review of Human Carcinogens;. 2012;100C:219-309.
188. Pira E, Donato F, Maida L, Discalzi G. Exposure to asbestos: past, present and future. Journal of thoracic disease. 2018;10(Suppl 2):S237-s45.
189. Frank AL, Joshi TK. The global spread of asbestos. Annals of global health. 2014;80(4):257-62.
190. Berman DW, Crump KS. Update of potency factors for asbestos-related lung cancer and mesothelioma. Critical reviews in toxicology. 2008;38 Suppl 1:1-47.
191. Markowitz SB, Levin SM, Miller A, Morabia A. Asbestos, asbestosis, smoking, and lung cancer. New findings from the North American insulator cohort. American journal of respiratory and critical care medicine. 2013;188(1):90-6.
192. Mossman BT, Churg A. Mechanisms in the pathogenesis of asbestosis and silicosis. American journal of respiratory and critical care medicine. 1998;157(5 Pt 1):1666-80.
193. Sen D. Working with asbestos and the possible health risks. Occupational medicine (Oxford, England). 2015;65(1):6-14.
194. Yap TA, Aerts JG, Popat S, Fennell DA. Novel insights into mesothelioma biology and implications for therapy. Nature reviews Cancer. 2017;17(8):475-88.
195. Bard MP, Hegmans JP, Hemmes A, Luider TM, Willemsen R, Severijnen LA, et al. Proteomic analysis of exosomes isolated from human malignant pleural effusions. American journal of respiratory cell and molecular biology. 2004;31(1):114-21.
196. Hegmans JP, Bard MP, Hemmes A, Luider TM, Kleijmeer MJ, Prins JB, et al. Proteomic analysis of exosomes secreted by human mesothelioma cells. The American journal of pathology. 2004;164(5):1807-15.
197. Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nature medicine. 2001;7(3):297-303.
198. Clayton A, Tabi Z. Exosomes and the MICA-NKG2D system in cancer. Blood cells, molecules & diseases. 2005;34(3):206-13.
199. Mahaweni NM, Kaijen-Lambers ME, Dekkers J, Aerts JG, Hegmans JP. Tumour-derived exosomes as antigen delivery carriers in dendritic cell-based immunotherapy for malignant mesothelioma. Journal of extracellular vesicles. 2013;2.

200. Thayanithy V, Babatunde V, Dickson EL, Wong P, Oh S, Ke X, et al. Tumor exosomes induce tunneling nanotubes in lipid raft-enriched regions of human mesothelioma cells. Experimental cell research. 2014;323(1):178-88.
201. Heusermann W, Hean J, Trojer D, Steib E, von Bueren S, Graff-Meyer A, et al. Exosomes surf on filopodia to enter cells at endocytic hot spots, traffic within endosomes, and are targeted to the ER. The Journal of cell biology. 2016;213(2):173-84.
202. Delage E, Cervantes DC, Penard E, Schmitt C, Syan S, Disanza A, et al. Differential identity of Filopodia and Tunneling Nanotubes revealed by the opposite functions of actin regulatory complexes. Sci Rep. 2016;6:39632.
203. Greening DW, Ji H, Chen M, Robinson BW, Dick IM, Creaney J, et al. Secreted primary human malignant mesothelioma exosome signature reflects oncogenic cargo. Sci Rep. 2016;6:32643.
204. Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nature. 2015;523(7559):177-82.
205. Creaney J, Dick IM, Leon JS, Robinson BW. A Proteomic Analysis of the Malignant Mesothelioma Secretome Using iTRAQ. Cancer genomics & proteomics. 2017;14(2):103-17.
206. Munson P, Lam YW, Dragon J, MacPherson M, Shukla A. Exosomes from asbestos-exposed cells modulate gene expression in mesothelial cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2018:fj201701291RR.
207. Bruno R, Poma AM, Alì G, Giannini R, Puppo G, Melfi F, et al. Novel prognostic markers for epithelioid malignant pleural mesothelioma. Journal of Clinical Oncology. 2017;35(15_suppl):e20028-e.
208. Roe OD, Anderssen E, Sandeck H, Christensen T, Larsson E, Lundgren S. Malignant pleural mesothelioma: genome-wide expression patterns reflecting general resistance mechanisms and a proposal of novel targets. Lung cancer (Amsterdam, Netherlands). 2010;67(1):57-68.
209. Munson P, Lam YW, MacPherson M, Beuschel S, Shukla A. Mouse serum exosomal proteomic signature in response to asbestos exposure. Journal of cellular biochemistry. 2018.
210. Ghio AJ, Stonehuerner J, Richards J, Devlin RB. Iron homeostasis in the lung following asbestos exposure. Antioxidants & redox signaling. 2008;10(2):371-7.
211. Pass HI, Levin SM, Harbut MR, Melamed J, Chiriboga L, Donington J, et al. Fibulin-3 as a Blood and Effusion Biomarker for Pleural Mesothelioma. The New England journal of medicine. 2012;367(15):1417-27.
212. Rajagopal C, Harikumar KB. The Origin and Functions of Exosomes in Cancer. Frontiers in oncology. 2018;8:66.
213. Whiteside TL. The emerging role of plasma exosomes in diagnosis, prognosis and therapies of patients with cancer. Contemporary oncology (Poznan, Poland). 2018;22(1a):38-40.

CHAPTER TWO

Mouse serum exosomal proteomic signature in response to asbestos exposure

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Abstract

Asbestos-induced diseases like fibrosis and mesothelioma are very aggressive, without any treatment options. These diseases are diagnosed only at the terminal stages due to lack of early stage biomarkers. The recent discovery of exosomes as circulating biomarkers led us to look for exosomal biomarkers of asbestos exposure in mouse blood. In our model, mice were exposed to asbestos as a single bolus dose by oropharyngeal aspiration. Fifty six days later blood was collected, exosomes were isolated from plasma and characterized and subjected to proteomic analysis using Tandem Mass Tag labelling. We identified many proteins, some of which were more abundant in asbestos exposed mouse serum exosomes, and three selected proteins were validated by immunoblotting. Our study is the first to show that serum exosomal proteomic signatures can reveal some important proteins relevant to asbestos exposure that have the potential to be validated as candidate biomarkers. We hope to extrapolate the positive findings of this study to humans in future studies.

The causative factor leading to the development of malignant mesothelioma (MM) is exposure to asbestos fibers [1]. Mesothelioma is a fatal cancer arising on the mesothelial cell lining of the pleura, most commonly, but can also present itself on the peritoneal lining, pericardium, and rarely the testicular tunica vaginalis. A recent CDC report states that there are a substantial number of MM cases, which are increasing in numbers [2]. The median lifespan, once diagnosed with MM, is 6-10 months and there are currently no successful treatment options. Further, there are no standard biomarkers for early diagnosis of the disease, neither are there any biomarkers to indicate harmful levels of asbestos exposure. It is therefore a highly valuable public health endeavor to identify signatures of asbestos exposure in order to more adequately detect harmful levels exposure before an individual develops MM.

The field of biomarker discovery has found its trajectory leading to the field of extracellular vesicle investigations, particularly exosomes. Exosomes are 30-140 nm membrane bound vesicles derived from endocytic origin, which are now known to be major players in transmitting biological content between cells and tissues [3-4]. Their content is remarkable in that it can be utilized for discovering unique biomarkers of disease states, such as cancer. Beyond biomarker discovery, exosome research has furthered the understanding of myriad biological mechanisms. Therefore, this avenue of discovery is fitting to make the necessary strides in being able to one day diagnose dangerous exposure to asbestos and pre-empt the development of MM.

To date there is no study of exosomes proteomic signature in relationship to asbestos exposure, making our paper the first of its kind. There is mounting evidence, however, of

the relevance and toxicology of exosomes released after exposure to chemicals and environmental toxins [5]. Asbestos itself is one of the most well-known environmental toxins and is currently classified as a Class I Carcinogen. Furthermore, the use of exosomes as cancer and disease biomarkers is now very common place, with serum exosomal proteomic signature being a particular focus for many studies on elucidating unique signatures [6-7]. One of the most exciting uses of serum exosomes for biomarker discovery was by Melo et al. in 2015 indicating that exosomal Glipican-1 was a discriminate factor for pinpointing pancreatic cancer [8].

The current study presented focuses on describing the exosomal proteomic analysis of an asbestos exposure mouse model. Our preliminary goal was to quarry for differential abundances of proteins in exosomes derived from asbestos exposed animals, particularly proteins in increased abundance, or unique, as those would more likely lead to future biomarker identification studies. The mouse model used here, exposes animals to asbestos via oropharyngeal aspiration (OA) in a bolus dose. OA is a well-established mode of exposing mice to asbestos fibers by deposition in the airway and lungs [9] and is closest to an inhalation model. Serum was collected and exosomes were isolated for proteomic profiling.

Although the full nature of exosome cargo packaging is not fully understood, it is known that exosomes become specifically enriched in certain proteins and molecules from the producer cell cytoplasm in ratios not directly parallel to the cytosolic fraction or freely secreted portion of those same molecules (given that all of them are freely secreted from the cell in the first place). Hence, isolating exosomes provides a specific vantage point that

avoids much of the other potentially unimportant molecules that are secreted into the circulation or into cavity spaces.

Materials and Methods

Oropharyngeal Aspiration of asbestos

C57/Bl6 mice (5/group) were exposed to crocidolite asbestos (NIEHS reference sample) or saline (50 μL) as a single bolus dose by oropharyngeal aspiration (OA) as described [9]. After 56 days of exposure, whole blood was collected via cardiac puncture and serum was collected using Microtainer Serum Separating Tubes (BD, Franklin Lakes, NJ) following the manufacturer's protocol. Serum was frozen at -80°C until exosome collection. All experiments using mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Vermont, Larner College of Medicine (Burlington, VT).

Exosome Isolation

Exosomes were collected from serum using ExoQuick (System BioSciences, Palo Alto, CA) according to the manufacturer's protocol [10-11]. Precipitation method was used for serum exosome isolation, because of very low volume (200 μL) of the serum sample availability.

Transmission Electron Microscopy (TEM)

The membranous structure and size of exosomes was assessed by TEM. Formvar/carbon coated nickel 200 mesh grids were glow discharged for 60 seconds, and 5μL of sample was placed on grid and incubated for 1 minute. Excess sample was wicked and the grids were touched to 30μL water drops with wicking performed between each rinse. Grids were

touched to 2 sequential 30µL drops of 2% aqueous uranyl acetate, excess was wicked, and grids were air dried. Grids were imaged under transmission electron microscope for exosomes using a JEOL 1400 TEM (JEOL, Peabody, MA).

NanoParticle Tracking Analysis

Number and size of exosomes were further assessed by nanoparticle tracking analysis (NTA) using the ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and Software ZetaView 8.02.31.

Exosome marker characterization

Exosome purity was characterized by assessment of exosome specific markers, CD9 and CD81 by using specific antibodies (Sigma Aldrich, St. Louis MO) in immunoblot analysis as described below [12].

Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and trypsin digestion

The extracted exosomal proteins (maximum amounts less than 100 µg) were loaded onto SDS-PAGE. Equal amounts (100 ng) of gylceraldehyde-3-phosphate dehydrogenase (Sigma Aldrich) were added to each sample to control for digestion and labeling efficiencies. The proteins were allowed to migrate 3 to 5-mm into the separating gel, and then the gels were stained with Coomassie Brilliant Blue. The gel lanes were excised into 3 slices according to their molecular weights (I- upper, II-mid, III-lower). (Figure 2A) The slices were destained with 50% acetonitrile (ACN)/50 mM NH_4HCO_3 and subjected to trypsin digestion protocols, as described previously [13].

After our initial pilot experiments, we have optimized this gel based separation strategy to allow the high abundant proteins to be confined to gel slice II-mid, whereas the relatively

low abundant proteins were localized in gel slices I-upper and III-lower. Gel slices I, II, II were analyzed separately in three mass spectrometry runs to increase the proteome coverage.

Peptide labeling by Tandem Mass Tags

The labeling procedures were performed according to the manufacturers' protocols (Thermo Fisher Scientific, Waltham, MA, USA) with the following modifications. Briefly, dried peptides in gel slice II, and gel slices I and III from each sample were resuspended in 50 and 25 µL of triethyl ammonium bicarbonate, respectively. Twenty and ten µL of TMT reagents (0.8 mg dissolved in 41 µL of acetonitrile (CH_3CN)) was added to gel slice II, and gel slices I and III, respectively, followed by briefly vortexing and an incubation for 1.5 h at room temperature. After incubation, 5% hydroxylamine was added to quench the reactions. One-third the reactions were combined, dried down and kept at -80^{o} C until mass spectrometry analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The purified labeled peptides were resuspended in 5 µL of 2.5% acetonitrile CH_3CN and 2.5% formic acid (FA) in water for subsequent LC-MS/MS based peptide identification and quantification. Analyses were performed on the Q-Exactive mass spectrometer coupled to an EASY-nLC (Thermo Fisher Scientific, Waltham, MA, USA). Samples were loaded onto a 100 µm x 120 mm capillary column packed with Halo C18 (2.7 µm particle size, 90 nm pore size, Michrom Bioresources, CA, USA) at a flow rate of 300 nl min-1. Peptides were separated using a gradient of 2.5-35% CH_3CN/0.1% FA over 150 min, 35-100% CH_3CN/0.1% FA in 1 min and then 100% CH_3CN /0.1% FA for 8 min, followed by

an immediate return to 2.5% CH_3CN/0.1% FA and a hold at 2.5% CH_3CN/0.1% FA. Peptides were introduced into the mass spectrometer via a nanospray ionization source and a laser pulled ~3 μm orifice with a spray voltage of 2.0 kV. Mass spectrometry data was acquired in a data-dependent "Top 10" acquisition mode with lock mass function activated (m/z 371.1012; use lock masses: best; lock mass injection: full MS), in which a survey scan from m/z 350-1600 at 70, 000 resolution (AGC target 1e6; max IT 100 ms; profile mode) was followed by 10 higher-energy collisional dissociation (HCD) tandem mass spectrometry (MS/MS) scans on the most abundant ions at 35,000 resolution (AGC target 1e5; max IT 100 ms; profile mode). MS/MS scans were acquired with an isolation width of 1.2 m/z and a normalized collisional energy of 35%. Dynamic exclusion was enabled (peptide match: preferred; exclude isotopes: on; underfill ratio: 1%; exclusion duration: 30 sec). Product ion spectra were searched using the SEQUEST and Mascot search engines on Proteome Discoverer 1.4 (Thermo Fisher Scientific, Waltham, MA, USA) against a curated Mouse Uniprot (Mus protein database; 3AUP000000589; downloaded Feb. 21, 2017) with sequences in forward and reverse orientations. Search Parameters were as follows: (1) full trypsin enzymatic activity; (2) maximum missed cleavages = 2; (3) minimum peptide length = 6, (4) mass tolerance at 20 ppm for precursor ions and 0.02 Da for fragment ions; (5) dynamic modifications on methionines (+15.9949 Da: oxidation), Dynamic TMT6plex modification (The TMT6plex and TMT10plex have the same isobaric mass) on N-termini and lysines (229.163 Da); (6) 4 maximum dynamic modifications allowed per peptide; and (7) static carbamidomethylation modification on cysteines (+57.021 Da). Percolator node was included in the workflow to limit the false positive

(FP) rates to less than 1% in the data set. The TMT ratios were generated with a common denominator using the four controls. All the protein identification and quantification information (<1% FP; with protein grouping enabled) was exported from the msf result files to Excel spreadsheets for further statistical analyses. Identification of keratins were removed from the list. Average means and p-values were calculated in Excel (Supplementary Table. S 1, Excel spreadsheet).

Western blot analysis for validation of proteins:

Few selected high abundance proteins were validated by immunoblot analysis in exosomes isolated from serum of saline and asbestos exposed mice. Western blot analysis was performed on exosome samples from serum suspended in 4X lysis buffer and boiled for 5 min at 95 °C. Thereafter 10–15 μL of each sample was resolved on a 10% SDS PAGE for subsequent immunoblotting for selected proteins, ceruloplasmin (Abcam, Cambridge, MA), haptoglobin (Abcam) and fibulin-1 (Thermo Fisher Scientific) using specific antibodies as described before [12].

Results

Characterization of exosomes

Mouse serum exosomes were purified by ExoQuick precipitation following the manufacturer's protocol and characterized before proteomics analysis. Isolated exosomes from mouse serum was initially characterized by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) to assess the particle concentration, size distribution, and membrane bound nature, as exosomes under TEM have a characteristic

cup-shaped morphology [14] (Figure 1A). NTA indicates particles directly the in size range of exosomes with a median size of 68.1nm (Figure 1B). Exosome preparation from serum also showed exosome specific markers, CD9 and CD81 (Figure 1C).

Proteomic analysis of exosomes

The exosomes isolated from serum and analyzed by Tandem-Mass-Tag profiling yielded results of 376 quantifiable proteins (with less than 1% FP). The proteins were compared between the control, non-asbestos exposed mice (n=4), and mice exposed to asbestos via pharyngeal aspiration (n=5) and sorted by fold change (asbestos/control) and statistical significance (Supplementary Table S1). Although there were only a few proteins that had differentially abundance in the asbestos group with statistical significance ($p < 0.05$) due to biological variations among individual animals, proteins were clearly more abundant after asbestos exposure (Figure 2B). The proteins identified to be more abundant in the asbestos group were sorted through for biological significance, as well as if there was statistical significance, and the top 15 which met either or both criteria were compiled (Table 1).

Validation of selected proteins by immunoblot analysis:

Three of these exosomal proteins in greater abundance from the asbestos exposed animals, and of particular biological interest (excluding any contaminating serum proteins), were validated by Western blot analysis to confirm proteomics results (Figure 3A), and ponceau staining was used to ensure equal protein loading (Figure 3B) as no reliable standard exists for secreted proteins. Four of five asbestos exposed animals showed increased exosomal fibulin-1, all five animals showed increased exosomal ceruloplasmin, and three of five

mice showed increased exosomal haptoglobin (Figure 3). Our protein validation was performed on the same group of mice and shows the same trends of abundance as indicated by our proteomics heatmap. The fact that the ratios determined by Western blot are in agreement with the TMT ratios for individual animals, demonstrates that our proteomics approach was robust and is applicable for these types of serum exosome proteomic profiling.

STRING Pathway Analysis

Pathway analysis was completed for the top 200 most abundant exosomal proteins in each group, based on fold change, using STRING functional protein-protein association network (https://string-db.org/) and the resulting top 10 Gene Ontology biological processes derived from the networking were compiled (Table 2).

Discussion

This short study is the first of its kind to present data on the relevance of exosome-protein signature in regard to in vivo exposure to asbestos fibers. Asbestos exposure is the main causal factor of MM, the fatal cancer of the mesothelial lining of the pleura, peritoneum, and pericardium. There is no means of early diagnosis of MM, or capability to diagnose harmful exposure to asbestos fibers due to a lack of useful biomarkers. We conducted these studies to begin in an unexplored area of asbestos and exosome biomarker discovery. We chose to use a well-defined mouse model of asbestos exposure, OA. Studies from NIOSH and others have compared OA with inhalation exposure to asbestos and found these two

very comparable ways to expose mice to asbestos [15-16]. OA is notably capable of eliciting the systemic effects of asbestos exposure from which we isolated serum exosomes.

Use of mouse models to understand the systemic and/or local effect of asbestos exposure on exosomal protein candidates is the first step towards identifying biomarkers of asbestos exposure. Our mouse model allowed us an *in vivo* approach to describing the exosomal protein content of mouse serum, and any associated differences upon asbestos exposure. Currently, the 2 most common methods available to isolate exosomes from various samples are ultracentrifugation and ExoQuick precipitation. Comparative exosome isolation studies done using these two methods showed either comparable results [11] or better [10] results with ExoQuick. Due to very small volume (~200 μL) of serum availability from mouse, we used the ExoQuick precipitation method to isolate exosomes. Characterization of our exosome preparation showed a membranous structure in correct size range expressing specific markers. Proteomic analysis of our preparation identified a total of 376 proteins. Amongst these proteins, we observed an increased abundance of multiple proteins of biological interest in the asbestos group. The heterogeneous effects observed in the asbestos group is not uncommon due to several reasons, 1) it is an insoluble (fibrous) agent and is not available to all cells (or all surfaces of tissues) uniformly, and 2) it is well known that only a small percentage of asbestos-exposed individuals develop mesothelioma suggesting a susceptibility issue [17-18].

Moreover, as this study was intended to yield insight on potential exosomal protein biomarkers for asbestos exposure we believe the proteins with most increased abundance

for asbestos exposed animals are of most interest. Within this subset of identified exosomal proteins there are some potential implications in the biology of asbestos exposure.

Increased proteins in the asbestos group that were validated by Western blot analysis were, ceruloplasmin, haptoglobin, and fibulin-1. These are acute phase proteins and shown previously to be upregulated in response to asbestos exposure [19-22]. Ceruloplasmin is a copper-carrying glycoprotein and plays an important role in iron metabolism [23-24]. The toxicity of asbestos is in part due to iron metabolism dysregulation after exposure in the lung [25]. Ceruplasmin has been shown in a 2014 study to be increased in the serum of asbestos exposed individuals and even higher in those with mesothelioma [26]. Use of postoperative tetrathiomolybdate to deplete copper and ceruloplasmin in mesothelioma patients has been shown to be beneficial [27], suggesting a strong role of ceruloplsmin in MM tumorigenesis.

The fibulin family of proteins (fibulin-1, -2, -3, -4, -5) are known to share extensive molecular functional similarities and sequence homology [28-29], and recent publications have indicated that fibulin-3 levels may be indicative markers of asbestos exposure and mesothelioma [30-32]. Our experiments have identified that fibulin-1 is increased in exosomes from asbestos exposed animals, which may indicate a role of fibulin family member in extracellular matrix remodeling after asbestos exposure. What is unique about fibulin-1 in this study is that it appears to be specifically enriched in exosomes of asbestos exposed animals, whereas the other studies indicate freely secreted fibulin-3.

Another protein of interest that was detected in increased abundance in the asbestos groups was ficolin-1, a protein involved in cell morphogenesis and known to target fibrinogen.

Additionally, exosomal 14-3-3 protein sigma was increased in serum of asbestos exposed mice and it has been shown that this protein has extracellular functionality as an adaptor protein. The only known extracellular 14-3-3 proteins are secreted via exosomes, and have been shown to target the Wnt signaling pathway in target cells via the association of discheveled-2 [33]. Interestingly, 14-3-3 protein sigma also plays roles in matrix metalloproteinase activity, activating fibroblast migration, and even reducing fibrosis and inflammation, both of which are hallmark effects of asbestos exposure [34]. Interestingly, 14-3-3-theta levels were found to be upregulated in conditioned medium from MM cells as compared to mesothelial cells [35], suggesting important role(s) of this group of proteins in mesothelioma tumorigenesis.

The potential consequences of exosomal proteins listed above is speculative based on previous research and requires further study to elucidate mechanistic roles. However, our data is novel by being the first to indicate unique protein signatures of exosomes in response to asbestos exposure. Those exosomal proteins in greater abundance after asbestos exposure may lead to the identification of more useful biomarkers to diagnose and prevent asbestos related disease, as exosomal strategies are becoming convenient and commonplace.

The development of useful biomarker-based diagnostic, and potentially therapeutic enterprises is of great public health concern for asbestos-related diseases and beyond. We intend to continue our expedition to mine for exosomal biomarkers of asbestos exposure in future and ongoing experiments. Those include *in vitro* models of asbestos exposure and isolation of exosomes from the blood of individuals with known exposure to asbestos and

mesothelioma patients. Our intentions are to provide new and useful exosome based strategies to identify asbestos exposure, and this study is the first of its kind in taking that initial step forward.

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Figures and Figure Legends

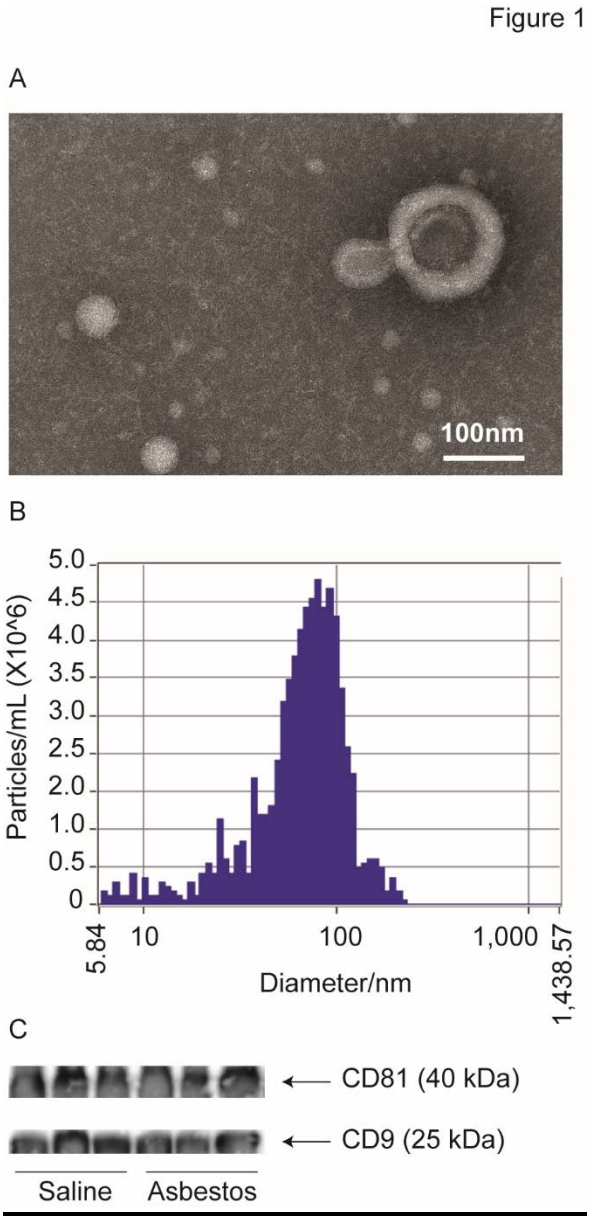

Figure 1. Characterization of exosomes derived from mouse serum by transmission electron microscopy (A), nanoparticle tracking analysis (B) and exosome specific marker immunoblotting (C).

Figure 2

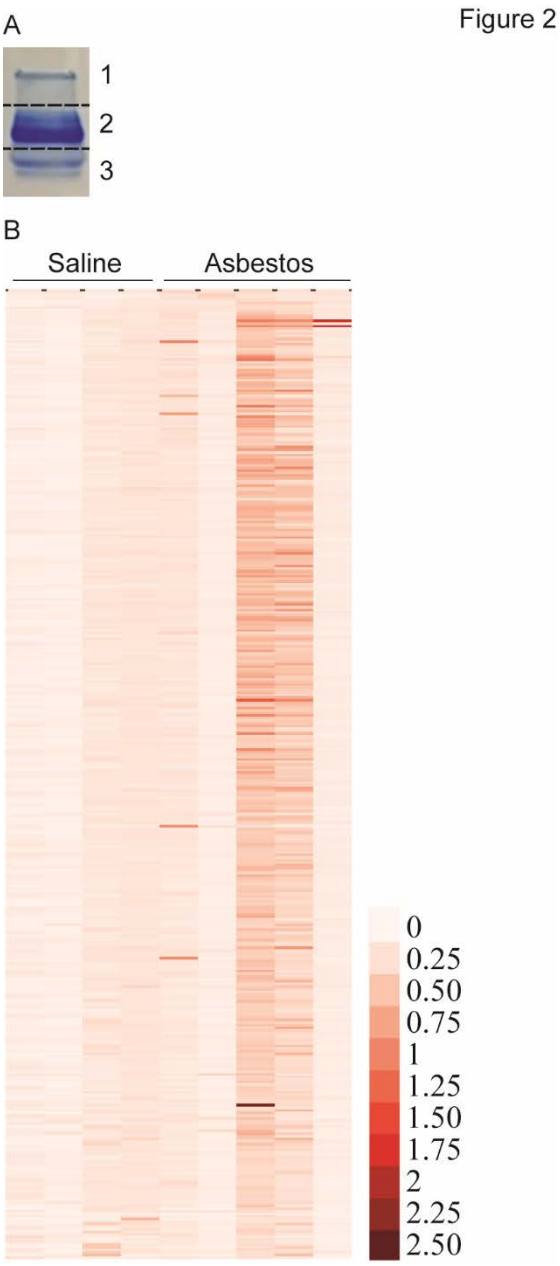

Figure 2. Depiction of gel cuts into three bands prior to in-gel trypsin digestion (A) and heatmap showing differential abundance of mouse serum exosomal proteins in response to asbestos exposure. Mice were exposed to saline (n=4) or asbestos (n=5) by oropharyngeal aspiration. Eight weeks later serum was collected, exosomes were isolated and proteomic analysis was performed as described in material and method section (B).

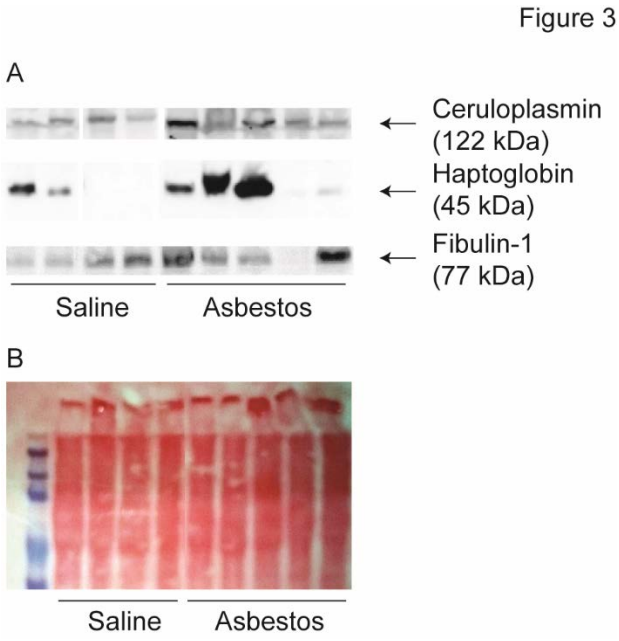

Figure 3. Proteomic results were validated by immunoblot assay. Three selected proteins, ceruloplasmin, haptoglobin, and fibulin, found in high abundance in asbestos group were validated by immunoblot analysis as described in materials and methods (A). As these are secreted proteins, loading controls are not available. Equal loading was achieved by keeping the starting and final volume same across the samples and was assessed by Ponceau staining after transferring proteins on membranes (B).

Table 1: Top 15 most biologically significant serum proteins from the most abundant exosomal proteins identified by proteomics analysis on asbestos-exposed mice compared to non-exposed mice

Accession #	Description	Fold Change	p-value
Q61646	Haptoglobin OS=Mus musculus GN=Hp PE=1 SV=1 - [HPT_MOUSE]	3.559	0.232
O70456	14-3-3 protein sigma OS=Mus musculus GN=Sfn PE=1 SV=2 - [1433S_MOUSE]	3.401	0.415
Q5FW60	Major urinary protein 20 OS=Mus musculus GN=Mup20 PE=1 SV=1 - [MUP20_MOUSE]	3.075	0.188
Q8K0E8	Fibrinogen beta chain OS=Mus musculus GN=Fgb PE=1 SV=1 - [FIBB_MOUSE]	3.058	0.172
Q91X70	Complement component 6 OS=Mus musculus GN=C6 PE=1 SV=1 - [Q91X70_MOUSE]	3.014	0.151
Q91X72	Hemopexin OS=Mus musculus GN=Hpx PE=1 SV=2 - [HEMO_MOUSE]	2.592	0.235
Q91V57-3	Isoform 3 of N-chimaerin OS=Mus musculus GN=Chn1 - [CHIN_MOUSE]	2.280	0.034

Q08879-2	Isoform C of Fibulin-1 OS=Mus musculus GN=Fbln1 - [FBLN1_MOUSE]	2.248	0.160
Q61147	Ceruloplasmin OS=Mus musculus GN=Cp PE=1 SV=2 - [CERU_MOUSE]	2.169	0.199
G3X9T8	Ceruloplasmin OS=Mus musculus GN=Cp PE=1 SV=1 - [G3X9T8_MOUSE]	2.166	0.198
Q3V3K3	Putative uncharacterized protein OS=Mus musculus GN=Taok3 PE=1 SV=1 - [Q3V3K3_MOUSE]	1.942	0.044
Q08879	Fibulin-1 OS=Mus musculus GN=Fbln1 PE=1 SV=2 - [FBLN1_MOUSE]	1.801	0.227
P19091	Androgen receptor OS=Mus musculus GN=Ar PE=1 SV=1 - [ANDR_MOUSE]	1.642	0.047
E9Q5F6	Polyubiquitin-C (Fragment) OS=Mus musculus GN=Ubc PE=4 SV=1 - [E9Q5F6_MOUSE]	1.549	0.030
Q5U405	Transmembrane protease serine 13 OS=Mus musculus GN=Tmprss13 PE=2 SV=2 - [TMPSD_MOUSE]	1.493	0.020

Table 2: Top 10 most significant gene ontology biological components in serum based on pathway analysis from the top 200 proteins of highest expression (highest fold change) in exosomes from asbestos exposed mice.

Pathway ID	Biological Process	Observed Gene Count	FDR
A			
GO.0006952	**defense response**	22	4.45E-13
GO.0006956	**complement activation**	9	1.55E-12
GO.0006959	**humoral immune response**	10	7.91E-11
GO.0006953	acute-phase response	8	1.85E-10
GO.0045087	**innate immune response**	14	7.56E-10
GO.0052547	regulation of peptidase activity	13	1.06E-08
GO.0006950	**response to stress**	27	1.33E-08
GO.0002253	activation of immune response	10	1.54E-08
GO.0030162	regulation of proteolysis	15	4.33E-08
GO.0052548	regulation of endopeptidase activity	12	5.87E-08

References

1. Berman, D. W.; Crump, K. S., Update of potency factors for asbestos-related lung cancer and mesothelioma. *Critical reviews in toxicology* **2008,** *38 Suppl 1*, 1-47.
2. Mazurek, J. M.; Syamlal, G.; Wood, J. M.; Hendricks, S. A.; Weston, A., Malignant Mesothelioma Mortality - United States, 1999-2015. *MMWR. Morbidity and mortality weekly report* **2017,** *66* (8), 214-218.
3. Colombo, M.; Raposo, G.; Thery, C., Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annual review of cell and developmental biology* **2014,** *30*, 255-89.
4. Munson, P.; Shukla, A., Exosomes: Potential in Cancer Diagnosis and Therapy. *Medicines* **2015,** *2* (4), 310-327.
5. Harischandra, D. S.; Ghaisas, S.; Rokad, D.; Kanthasamy, A. G., Exosomes in Toxicology: Relevance to Chemical Exposure and Pathogenesis of Environmentally Linked Diseases. *Toxicological sciences : an official journal of the Society of Toxicology* **2017**.
6. Sun, Y.; Liu, S.; Qiao, Z.; Shang, Z.; Xia, Z.; Niu, X.; Qian, L.; Zhang, Y.; Fan, L.; Cao, C. X.; Xiao, H., Systematic comparison of exosomal proteomes from human saliva and serum for the detection of lung cancer. *Analytica chimica acta* **2017,** *982*, 84-95.
7. Colletti, M.; Petretto, A.; Galardi, A.; Paolo, V. D.; Tomao, L.; Lavarello, C.; Inglese, E.; Bruschi, M.; Lopez, A. A.; Pascucci, L.; Geoerger, B.; Peinado, H.; Franco, L.; Angela, D. G., Proteomic analysis of neuroblastoma-derived exosomes: new insights into a metastatic signature. *Proteomics* **2017**.
8. Melo, S. A.; Luecke, L. B.; Kahlert, C.; Fernandez, A. F.; Gammon, S. T.; Kaye, J.; LeBleu, V. S.; Mittendorf, E. A.; Weitz, J.; Rahbari, N.; Reissfelder, C.; Pilarsky, C.; Fraga, M. F.; Piwnica-Worms, D.; Kalluri, R., Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* **2015,** *523* (7559), 177-82.
9. Teeguarden, J. G.; Webb-Robertson, B. J.; Waters, K. M.; Murray, A. R.; Kisin, E. R.; Varnum, S. M.; Jacobs, J. M.; Pounds, J. G.; Zanger, R. C.; Shvedova, A. A., Comparative proteomics and pulmonary toxicity of instilled single-walled carbon nanotubes, crocidolite asbestos, and ultrafine carbon black in mice. *Toxicological sciences : an official journal of the Society of Toxicology* **2011,** *120* (1), 123-35.
10. Eitan, E.; Green, J.; Bodogai, M.; Mode, N. A.; Baek, R.; Jorgensen, M. M.; Freeman, D. W.; Witwer, K. W.; Zonderman, A. B.; Biragyn, A.; Mattson, M. P.; Noren Hooten, N.; Evans, M. K., Age-Related Changes in Plasma Extracellular Vesicle Characteristics and Internalization by Leukocytes. *Sci Rep* **2017,** *7* (1), 1342.
11. Helwa, I.; Cai, J.; Drewry, M. D.; Zimmerman, A.; Dinkins, M. B.; Khaled, M. L.; Seremwe, M.; Dismuke, W. M.; Bieberich, E.; Stamer, W. D.; Hamrick, M. W.; Liu, Y., A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLoS One* **2017,** *12* (1), e0170628.
12. Thompson, J. K.; MacPherson, M. B.; Beuschel, S. L.; Shukla, A., Asbestos-Induced Mesothelial to Fibroblastic Transition Is Modulated by the Inflammasome. *The American journal of pathology* **2017,** *187* (3), 665-678.

13. Hristova, M.; Veith, C.; Habibovic, A.; Lam, Y. W.; Deng, B.; Geiszt, M.; Janssen-Heininger, Y. M.; van der Vliet, A., Identification of DUOX1-dependent redox signaling through protein S-glutathionylation in airway epithelial cells. *Redox biology* **2014,** *2*, 436-46.
14. Szatanek, R.; Baj-Krzyworzeka, M.; Zimoch, J.; Lekka, M.; Siedlar, M.; Baran, J., The Methods of Choice for Extracellular Vesicles (EVs) Characterization. *International journal of molecular sciences* **2017,** *18* (6).
15. Mercer, R. R.; Hubbs, A. F.; Scabilloni, J. F.; Wang, L.; Battelli, L. A.; Friend, S.; Castranova, V.; Porter, D. W., Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes. *Part Fibre Toxicol* **2011,** *8*, 21.
16. Porter, D. W.; Hubbs, A. F.; Mercer, R. R.; Wu, N.; Wolfarth, M. G.; Sriram, K.; Leonard, S.; Battelli, L.; Schwegler-Berry, D.; Friend, S.; Andrew, M.; Chen, B. T.; Tsuruoka, S.; Endo, M.; Castranova, V., Mouse pulmonary dose- and time course-responses induced by exposure to multi-walled carbon nanotubes. *Toxicology* **2010,** *269* (2-3), 136-47.
17. Roushdy-Hammady, I.; Siegel, J.; Emri, S.; Testa, J. R.; Carbone, M., Genetic-susceptibility factor and malignant mesothelioma in the Cappadocian region of Turkey. *Lancet* **2001,** *357* (9254), 444-5.
18. Mitchell Cheung, C. W. M. a. J. R. T., Germline and somatic mutations in human mesothelioma and lessons from asbestos-exposed genetically engineered mouse models. In *Asbestos and Mesothelioma*, Testa, J. R., Ed. Springer: 2017; pp 175-196.
19. Shannahan, J. H.; Alzate, O.; Winnik, W. M.; Andrews, D.; Schladweiler, M. C.; Ghio, A. J.; Gavett, S. H.; Kodavanti, U. P., Acute phase response, inflammation and metabolic syndrome biomarkers of Libby asbestos exposure. *Toxicology and applied pharmacology* **2012,** *260* (2), 105-14.
20. Ke, F.; Zhang, J.; Jin, S., [The relationship of biochemical changes among bronchoalveolar lavage fluid serum and lung on dust-exposed rats]. *Hua xi yi ke da xue xue bao = Journal of West China University of Medical Sciences = Huaxi yike daxue xuebao* **1991,** *22* (3), 292-5.
21. Spitsyn, V. A.; Tsurikova, G. V.; Afanas'eva, I. S., [Gene manifestations in negative man-made environment: a selective genetically determined sensitivity to asbestos]. *Vestnik Rossiiskoi akademii meditsinskikh nauk* **1992,** (4), 46-52.
22. Afanas'eva, I. S.; Spitsyn, V. A.; Tsurikova, G. V., [Genetic polymorphism of haptoglobin and quantitative changes in its levels during exposure to asbestos]. *Genetika* **1993,** *29* (11), 1895-900.
23. Frieden, E., Ceruloplasmin, A Link Between Copper and Iron Metabolism. In *Bioinorganic Chemistry*, AMERICAN CHEMICAL SOCIETY: 1971; Vol. 100, pp 292-321.
24. Roeser, H. P.; Lee, G. R.; Nacht, S.; Cartwright, G. E., The role of ceruloplasmin in iron metabolism. *Journal of Clinical Investigation* **1970,** *49* (12), 2408-2417.
25. Ghio, A. J.; Stonehuerner, J.; Richards, J.; Devlin, R. B., Iron homeostasis in the lung following asbestos exposure. *Antioxidants & redox signaling* **2008,** *10* (2), 371-7.
26. Sezgi, C.; Taylan, M.; Selimoglu Sen, H.; Evliyaoğlu, O.; Kaya, H.; Abakay, O.; Abakay, A.; Tanrıkulu, A. C.; Senyiğit, A., Oxidative Status and Acute Phase Reactants in Patients with Environmental Asbestos Exposure and Mesothelioma. *The Scientific World Journal* **2014,** *2014*, 902748.
27. Pass, H. I.; Brewer, G. J.; Dick, R.; Carbone, M.; Merajver, S., A phase II trial of tetrathiomolybdate after surgery for malignant mesothelioma: final results. *The Annals of thoracic surgery* **2008,** *86* (2), 383-9; discussion 390.

28. Kobayashi, N.; Kostka, G.; Garbe, J. H.; Keene, D. R.; Bachinger, H. P.; Hanisch, F. G.; Markova, D.; Tsuda, T.; Timpl, R.; Chu, M. L.; Sasaki, T., A comparative analysis of the fibulin protein family. Biochemical characterization, binding interactions, and tissue localization. *J Biol Chem* **2007,** *282* (16), 11805-16.
29. Timpl, R.; Sasaki, T.; Kostka, G.; Chu, M.-L., Fibulins: a versatile family of extracellular matrix proteins. *Nat Rev Mol Cell Biol* **2003,** *4* (6), 479-489.
30. Pass, H. I.; Levin, S. M.; Harbut, M. R.; Melamed, J.; Chiriboga, L.; Donington, J.; Huflejt, M.; Carbone, M.; Chia, D.; Goodglick, L.; Goodman, G. E.; Thornquist, M. D.; Liu, G.; de Perrot, M.; Tsao, M.-S.; Goparaju, C., Fibulin-3 as a Blood and Effusion Biomarker for Pleural Mesothelioma. *N Engl J Med* **2012,** *367* (15), 1417-1427.
31. Kirschner, M. B.; Pulford, E.; Hoda, M. A.; Rozsas, A.; Griggs, K.; Cheng, Y. Y.; Edelman, J. J.; Kao, S. C.; Hyland, R.; Dong, Y.; Laszlo, V.; Klikovits, T.; Vallely, M. P.; Grusch, M.; Hegedus, B.; Dome, B.; Klepetko, W.; van Zandwijk, N.; Klebe, S.; Reid, G., Fibulin-3 levels in malignant pleural mesothelioma are associated with prognosis but not diagnosis. *Br J Cancer* **2015,** *113* (6), 963-9.
32. Rapisarda, V.; Caltabiano, R.; Musumeci, G.; Castrogiovanni, P.; Ferrante, M.; Ledda, C.; Lombardo, C.; Graziano, A. C. E.; Cardile, V.; Loreto, C., Analysis of fibulin-3 after exposure to asbestos-like fibers. *Environmental research* **2017,** *156*, 381-387.
33. Dovrat, S.; Caspi, M.; Zilberberg, A.; Lahav, L.; Firsow, A.; Gur, H.; Rosin-Arbesfeld, R., 14-3-3 and β-catenin are secreted on extracellular vesicles to activate the oncogenic Wnt pathway. *Mol Oncol* **2014,** *8* (5), 894-911.
34. Kaplan, A.; Bueno, M.; Fournier, A. E., Extracellular functions of 14-3-3 adaptor proteins. *Cellular signalling* **2017,** *31*, 26-30.
35. Creaney, J.; Dick, I. M.; Leon, J. S.; Robinson, B. W., A Proteomic Analysis of the Malignant Mesothelioma Secretome Using iTRAQ. *Cancer genomics & proteomics* **2017,** *14* (2), 103-117.

CHAPTER THREE

Exosomes from asbestos exposed cells modulate gene expression in mesothelial cells

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Abbreviations

MM: malignant mesothelioma

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TMT: tandem mass tag

PMA: phorbol myristate acetate

LC-MS/MS: liquid chromatography-tandem mass spectrometry

FA: formic acid

MS/MS: tandem mass spectrometry

NTA: nanoparticle tracking analysis

TEM: transmission electron microscopy

DLS: dynamic light scattering

EMT: epithelial to mesenchymal transition

CH_3CN: acetonitrile

HCD: higher-energy collisional dissociation

Abstract

Asbestos exposure is a determinate cause of many diseases such as mesothelioma, fibrosis and lung cancer posing it as an important human health hazard. At this time, there are no identified biomarkers to demarcate asbestos exposure prior to the presentation of disease and symptoms, and there is only limited understanding of the underlying biology that governs asbestos induced disease. Our study uses exosomes, 30-140 nm extracellular vesicles, to gain insight into these knowledge gaps. As inhaled asbestos is first encountered by lung epithelial cells and macrophages, we hypothesize that asbestos exposed cells secrete exosomes with signature proteomic cargo that can alter the gene expression of mesothelial cells, contributing to disease outcomes like mesothelioma. In the present study using lung epithelial cells (BEAS2B) and macrophages (THP-1), we first show that asbestos exposure causes changes in abundance of some proteins in the exosomes secreted from these cells. Furthermore, exposure of human mesothelial cells (HPM3) to these exosomes resulted in gene expression changes related to epithelial to mesenchymal transition and other cancer related genes. This is the first report to indicate that asbestos exposed cells secrete exosomes with differentially abundant proteins and that those exosomes have a genetically altering effect on mesothelial cells.

Key words: extracellular vesicles, mesothelioma, biomarkers, proteomics, tumorigenesis

Introduction

Exposure to asbestos is a main causal factor of several human diseases, including malignant mesothelioma (MM), lung fibrosis (asbestosis) and bronchial carcinoma (1). Notably, lung cancer risk is supra-additively increased when an individual both smokes tobacco and is exposed to asbestos (2). The term asbestos (stemming from the Greek term for inextinguishable) refers to a group of hydrated silicate fibers with a length-to-width ratio greater than 3 and is classified as a category 1 carcinogen (3). The wide use of asbestos for industrial purposes across the world, demonstrates its relevance as a human health hazard, now and for years to come (4). Exposure to asbestos occurs primarily through inhalation with fibers first making contact with the upper respiratory tract. Depending on fiber geometry, some asbestos fibers (such as those that are longer and thinner) will penetrate deeper into the lung, and tend to have more deleterious biological effects (5). The initial, and lasting, assault of asbestos occurs on airway epithelial cells and resident macrophages (6, 7), and because the mechanisms of asbestos related disease remains unclear, we hypothesize that these epithelial cells and macrophages exposed to asbestos secrete signature factors that contribute to disease development.

To date, there are no studies implicating the role of exosomes in MM pathogenesis and diagnosis. The latency period after initial exposure to developing malignant disease is 15-60 years, and once diagnosed, MM is fatal within 6-12 months (8). We believe that identifying a unique protein secretome from asbestos exposed cells will contribute to the advancement in knowledge needed to diagnose asbestos exposure and possibly aid in future clinical settings. Due to this need for biomarker identification, the aim of this current study

is to evaluate secreted protein signatures from *in vitro* asbestos exposure models by focusing on proteins found within the subset of extracellular vesicles known as exosomes. Exosomes are membrane bound vesicles in the size range of 30-140 nm, and are derived from endocytic origin (9). These secreted particles have emerged as attractive tools in biomarker identification and as tools for evaluating biological phenomena. Importantly, exosomes are now known to be more than simple waste disposal, but have vital roles in normal physiology and disease states (10). Identification of protein biomarkers using exosomes is gaining significant traction in the field of disease research, and has potential for uncovering new modes of diagnoses (11), similar to the discovery of glypican-1 containing exosomes in the identification of pancreatic cancer (12). Additionally, exosomes from MM cells and tumors have been previously quarried for their proteomic signature (13, 14), but this study will be the very first of its kind in identifying exosomal proteins from asbestos exposed human cells. This is an essential effort, because in order to have a more thorough understanding of this disease, we must delve into all aspects of asbestos exposure leading to disease development.

The purpose of this research study is to descriptively outline the protein subsets determined from exosome isolates of asbestos exposed cells, particularly those unique or upregulated as compared to non-asbestos exposed controls.

Furthermore, we are very interested in understanding the mechanism by which MM develops. As it is not clear whether mesothelial cells transform via direct interaction with asbestos fibers, or by secreted factors from other cells interacting with the fibers. Perhaps it is both, but we envisioned that exosomes may also be progenitors of disease by sending

molecular cargo to mesothelial cells, from asbestos exposed epithelial cells or macrophages, that may transform non-tumorigenic mesothelial cells to becoming more tumorigenic. This is a novel idea developed by our lab, that exosomes from asbestos exposed cells might lead to or prime disease in an unexposed region (i.e. the pleura or peritoneum).

We hypothesize that asbestos exposed cells secrete exosomes containing unique protein cargo that might be informative in the biology of asbestos related disease states, and that these exosomes are capable of biologically altering target mesothelial cells to becoming more tumorigenic.

Materials and Methods

Cell culture and treatment

Human bronchial epithelial cells (BEAS2B) and macrophage cell line (THP-1) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown as reported before (6, 15) in exosome-free fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA).

Cells were down-shifted to reducing medium (0.5% exosome free medium) for overnight and NIEHS reference sample crocidolite asbestos was added to the cells (5 $\mu g/cm^2$, 72 hr) as described previously (16). For THP-1 cells, we performed experiments with and without priming cells with the tumor promoting agent (TPA) phorbol myristate acetate (PMA) before adding asbestos. Cells were pre-treated with 0.5μM PMA for 3 hours as described previously(6). In present experiments, we used exosomes from untreated cells as control.

Inert particles were not used as we have shown in our previous publications that they don't have significant effect on gene expression or other biological processes (17-19).

After 72 hr incubation with asbestos or controls with no asbestos (and other treatments), conditioned cell culture supernatant was removed for exosome isolation.

Exosome Isolation from cell culture medium

Exosomes were isolated using ExoQuick-TC precipitation reagent (System BioSciences, Palo Alto, CA, USA), according to the manufacturer's protocol (20, 21), incorporating a 0.22μm filtration step after the first centrifugation to ensure a more pure yield of exosomes.

Transmission Electron Microscopy (TEM)

Formvar/carbon coated nickel 200 mesh grids were glow discharged for 60 seconds, and 5μl of sample was placed on grid and incubated for 1 minute. Excess sample was wicked and grid was touched to 30μl water drops with wicking performed between each rinse. Grid was touched to 2 sequential 30μl drops of 2% aqueous uranyl acetate, excess was wicked, and grids were air dried. Grids were imaged under transmission electron microscope (JEOL 1400 TEM) for exosomes.

Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were made on exosome preparations suspended in PBS using the Zetasizer Nano ZSP system Model ZEN5600 (Malvern Instruments, Malvern, United Kingdom) using a 633 nm He-Ne laser as the light source and the Malvern application software.

Nanoparticle Tracking Analysis

Exosomes number and size were further assessed by nanoparticle tracking analysis (NTA) using the ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and Software ZetaView 8.02.31.

Scanning electron microscopy for detection of asbestos fibers in exosomes

Exosomes from control or asbestos exposed cells were imaged using a JEOL 6060 scanning electron microscope to check for presence of asbestos fibers within exosomes.

Characterization of exosomes by antibodies: Few isolated exosome samples from experiment were characterized by anti-CD81

Two aliquots of isolated exosomes from representative groups were characterized by immunoblot analysis for presence of exosomal marker CD81 (Sigma Aldrich) and also for absence of calnexin (Novus Biologicals, Littleton, CO, USA) to rule out contaminating ER vesicles.

Exosome uptake by mesothelial cells

Exosomes from epithelial cells and macrophages were labeled using PKH67 dye (Sigma Aldrich) according to the manufacturer's protocol. Labeled exosomes were suspended in PBS and added to target mesothelial cells and imaged on an Olympus IX70 inverted light microscope.

Proteomic analysis on exosome samples

Proteins extracted from equal volume of medium (maximum amounts less than 100µg) were run by SDS-PAGE. Equal amounts (100ng) of gylceraldehyde-3-phosphate dehydrogenase (GAPDH, Sigma: G5537-100UN) were added to each sample to control for digestion and labeling efficiencies. The proteins were allowed to migrate 3 to 5mm into

the separating gel, which were stained with Coomassie Brilliant Blue. Single wide bands in which the proteins still had not been separated were excised, destained with 50% acetonitrile (CH_3CN)/50 mM NH_4HCO_3, and subjected to trypsin digestion protocols, as described previously (22).

Peptide labeling by Tandem Mass Tags

The labeling procedures were performed according to the manufacturers' protocols (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the dried peptides from each sample were resuspended in 102.5µL of triethyl ammonium bicarbonate, and 0.8 mg of TMT reagents dissolved in 41µL of CH_3CN was added, followed by briefly vortexing and an incubation for 1.5 hr at room temperature. After incubation, 8µL of 5% hydroxylamine was added to quench the reactions. Twenty-five µL from each of the reactions (control, PMA, asbestos, PMA and asbestos for THP-1 experiment, or control and asbestos for BEAS2B experiment) were combined and dried down. The THP-1 samples were further purified by ZipTip (Millipore, MA, USA). All samples were kept at -80^{o}C until mass spectrometry analysis.

Protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The purified labeled peptides were resuspended in 5µL of 2.5% CH_3CN and 2.5% formic acid (FA) in water for subsequent LC-MS/MS based peptide identification and quantification. Analyses were performed on the Q-Exactive mass spectrometer coupled to an EASYnLC (Thermo Fisher Scientific, Waltham, MA, USA). Samples were loaded onto a 100µm x 120mm capillary column packed with Halo C18 (2.7 µm particle size, 90

nm pore size, Michrom Bioresources, CA, USA) at a flow rate of 300nl min-1. Peptides were separated using a gradient of 2.5-35% CH_3CN/0.1% FA over 150 min, 35-100% CH_3CN/0.1% FA in 1 min and then 100% CH_3CN/0.1% FA for 8 min, followed by an immediate return to 2.5% CH_3CN/0.1% FA and a hold at 2.5% CH_3CN/0.1% FA. Peptides were introduced into the mass spectrometer via a nanospray ionization source and a laser pulled ~3 μm orifice with a spray voltage of 2.0 kV. Mass spectrometry data was acquired in a data-dependent "Top 10" acquisition mode with lock mass function activated (m/z 371.1012; use lock masses: best; lock mass injection: full MS), in which a survey scan from m/z 350-1600 at 70,000 resolution (AGC target 1e6; max IT 100 ms; profile mode) was followed by 10 higher-energy collisional dissociation (HCD) tandem mass spectrometry (MS/MS) scans on the most abundant ions at 35,000 resolution (AGC target 1e5; max IT 100 ms; profile mode). MS/MS scans were acquired with an isolation width of 1.2 m/z and a normalized collisional energy of 35%. Dynamic exclusion was enabled (peptide match: preferred; exclude isotopes: on; underfill ratio: 1%; exclusion duration: 30 sec). Product ion spectra were searched using the SEQUEST and Mascot search engines on Proteome Discoverer 2.2 (Thermo Fisher Scientific, Waltham, MA, USA) against a curated Human Uniprot (Homo sapiens protein database; 3AUP000005640; downloaded September 22, 2017). Common processing and consensus workflows for Reporter based Quantification were used with minor modifications. In the processing workflow, the following parameters were set as follows: (1) full trypsin enzymatic activity; (2) maximum missed cleavages = 2; (3) minimum peptide length = 6, (4) mass tolerance at 10 ppm for precursor ions and 0.02 Da for fragment ions; (5) dynamic modifications on methionines

(+15.9949 Da: oxidation), dynamic TMT6plex modification (The TMT6plex and TMT10plex have the same isobaric mass) on N-termini and lysines (229.163 Da); and (6) static carbamidomethylation modification on cysteines (+57.021 Da). Percolator node was included in the workflow to limit the false positive (FP) rates to less than 1% in the data set.

Statistical analysis

In the consensus workflow, parameters were set as follows: (1) both unique and razor peptides were used for quantification; (2) Reject Quan Results with Missing Channels: False; (3) Apply Quan Value Corrections: False; (4) Co-Isolation Threshold: 50; (5) Average Reporter S/N Threshold = 10; (6) "Total Peptide Amount" was used for normalization and (7) Scaling Mode was set "on All Average". Ratio calculation was Summed Abundance Based. For Hypothesis testing, "background based" ANOVA was used for analyzing the two independent experiments of THP-1 cells (two separate 4plex TMT runs (two SDS-PAGE), control, treated with asbestos, treated with PMA, treated both with PMA and asbestos) and the "individual proteins" ANOVA was used for the experiment of BEAS2B cells (two technical replicates were run for the 6plex TMT with 3 biological replicates, (control C1 -3, asbestos treated A1-3) incorporated). p-values and adjusted p-values (Benjamini-Hochberg method) were calculated accordingly. Only proteins identified in all replicates were kept. For THP1 data, fold changes (asbestos/control; PMA/control; PMA+asbestos /control) from the two biological replicates with CV% > 20% were eliminated.

All the protein identification and quantification information (<1% FP; with protein grouping enabled) was exported from the Proteome Discoverer result files to Excel spreadsheets for further statistical analyses. The normalized and scaled (to total peptide amount) values were then imported into the JMP Pro 13 (SAS Institute, Cary, North Carolina, USA) to construct the heat maps.

Validation of proteins by immunoblot analysis

Exosomal proteins were validated by immunoblot analysis using antibodies specific to vimentin (Cell Signaling, Danvers, MA, USA), thrombospondin, superoxide dismutase, and glypican-1 (Abcam Cambridge, MA, USA) as previously published (23). Proteins selected for validation were of biological relevance to asbestos exposure and/or cancer.

Exposure of human mesothelial cells to isolated exosomes

Primary human pleural mesothelial cells (HPM3) were purchased from Brigham and Women's Hospital, Boston and cultured as previously published (18). Exosomes isolated from asbestos exposed, or unexposed control cells (above) (either BEAS2B or THP-1) were suspended in PBS. For the BEAS2B exosome experiment, either 10µg or 20µg of exosome protein were added to target mesothelial cells every day for 4 days. For the macrophage experiment, equal volumes of exosome preparation rather than protein content from different groups (to take in consideration of different number of exosomes released per different conditions), were added to mesothelial cells. After 96 hr of treatment, mesothelial cells were harvested and total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hiden, Germany).

PCR Array to assess the effect of BEAS2B exosomes on EMT related genes in mesothelial cells:

Effect of BEAS2B exosomes on mesothelial cells was analyzed by PCR Array using EMT template (Qiagen) to assess the gene expression patterns of epithelial-to-mesenchymal transition (EMT) genes as previously described (23).

Microarray analysis to assess the effects of THP-1 exosomes on mesothelial cells:

RNA quality from THP-1 exosome exposed mesothelial cells was assessed prior to microarray analysis using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and subsequently the RNA was analyzed using the Clariom S assay (Thermo Fisher Scientific, Waltham, MA, USA) microarray for human samples. This was chosen for a wider breadth of potential gene expression changes outside of the more narrowed view of one pathway. Microarray data analysis was performed using the Transcriptome Analysis Console 4.0 (Thermo Fisher). Our parameters were set to any gene that was expressed differently by both 2 fold and 1.5 fold up or down with a p-value less than 0.05.

NIH DAVID was used to classify functional annotation and pathway analysis for genes that were expressed differently in our experimental groups.

QRT-PCR to validate gene expression changes:

Validation of expression changes in selected genes of interest (related to asbestos exposure and/or cancer) was conducted by qRTPCR after cDNA synthesis from 1µg RNA using Reverse Transcription Using AMV Reverse Transcriptase (Promega, Madison, WI, USA) following the manufacturer's protocol as previously published (23). We used Assays on

Demand primers and probes for *E-cadherin, IL-1ra, desmoplakin, CCNB2, EGR1, FANCD2, ERO1B, CRELD2* and *JAG1* (Thermo Fisher).

Results

Exosome isolation and characterization from BEAS2B cells

Successful isolation of exosomes was characterized by TEM, DLS, and Western blot analysis for exosomal marker CD81 (Figure 1). TEM indicates membrane bound vesicles in the size range indicative of exosomes along with the characteristic depressed spherical shape (or "cup-shaped" as some describe) of exosomes imaged by TEM. DLS show vesicle size populations in the size range of exosomes. SEM analysis showed no presence of asbestos fibers inside exosomes. No samples showed any significant signal for calnexin suggesting no contamination of ER.

Proteomic analysis of exosomes from asbestos exposed and control BEAS2B cells showed different signature

Proteomics profiling was conducted on exosomes from BEAS2B cells, asbestos exposed or control, using isobaric TMT tags. We identified a total of 145 proteins and compiled a list of proteins with significant differential abundances (55 proteins with p-value ≤ 0.05, and 34 proteins with adjusted p-value ≤ 0.05) in asbestos exposed group when compared to the control group (Table 1, Figure 2A, B).A few of such proteins increased in abundance in the asbestos group are: plasminogen activator inhibitor 1, vimentin, 14-3-3 protein sigma, thrombospondin, transitional endoplasmic reticulum ATPase, and glypican-1 (Table 1).

Two proteins identified by proteomic analysis were validated by immunoblot analysis

Western blot analysis of glypican-1 and thrombospondin validated that these exosomal proteins are increased from epithelial cells exposed to asbestos (Figure 2C). Due to secretory nature of exosome no normalization control could be included and equal loading of proteins was verified by Ponceau staining (data not shown).

Exosomes from asbestos exposed epithelial cells are taken up by mesothelial cells

A set of isolated exosomes were PKH67 labelled and added to mesothelial cells to verify that epithelial cell exosomes interact and are taken up by mesothelial cells. As shown in Figure 3A, green fluorescent labelled exosomes were identified inside mesothelial cells suggesting their uptake by mesothelial cells. Monitoring different areas of dish showed a consistent 50-60% cells positive for labelled exosomes.

Exosomes from asbestos exposed epithelial cells caused altered gene expression in mesothelial cells

In order to assess if exosomes from asbestos exposed epithelial cells were capable of altering the gene expression pattern of mesothelial cells, isolated exosomes from exposed, or control epithelial cells were added repeatedly to mesothelial cells. After 96 hr, mesothelial cell RNA was isolated and analyzed by PCR Array for EMT. The data indicated multiple gene changes, the top 10 most up- or down-regulated in response to exosomes from asbestos exposed cells are listed in Table 2. From this list, 3 genes were validated by qRTPCR (Figure 3B), E-cadherin, desmoplakin, and IL1 Receptor Antagonist (IL1RN). We observed a downward trend in these three genes in response to exposure to exosomes from asbestos exposed cells as compared to control exosomes (Figure 3B).

Exosomes isolated and characterized from THP-1

Exosomes from THP-1 cells were isolated and characterized for their purity, size and intactness using antibodies, NTA, DSL and TEM (Figure 1). DLS and NTA both show vesicle size populations in the size range of exosomes, with some discrepancies between samples analyzed on both machines. This anomaly could be explained by the differing nature of how each instrument analyzes particles and particles sizes, and perhaps the population of very small (~10 nm) vesicles may be an artifact of DLS, as this population is not observed with the more robust technique of NTA on the same sample. TEM data shows intact, membrane bound exosomes in size range of 40-140 nm (Figure 1). CD81 presence and calnexin absence demonstrated the purity of the preparation (Figure 1D).

Proteomic analysis showed increased abundance of proteins in asbestos exposed exosomes from THP-1

Exosomes were isolated from 4 different groups of THP-1 cells; control- non-asbestos exposed; Asbestos exposed; PMA primed- asbestos exposed; PMA primed- no asbestos exposure.

Proteomic analysis of TMT labeled macrophage exosomal proteins from all groups provided a total list of 785 identified exosomal proteins, many of which showing moderate alterations in abundance between groups (Figure 4A, Table 3). PMA priming had no added effect on exosomal protein signature as compared to asbestos alone (no priming) group, (data not shown) suggesting that unlike other stimuli, asbestos exposure to human macrophages do not require priming.

Thirty-two proteins were identified with differential abundance in the asbestos treated group as compared with the control group ($p < 0.05$; Figure 4B, Table 3). Fifteen proteins

were in greater abundance from asbestos exposed groups. Two of these proteins' abundance were validated by Western blot analysis to be in fact increased in exosomes from asbestos exposed macrophages: superoxide dismutase and vimentin (Figure 4C).

Exosomes from THP-1 cells are taken up by mesothelial cells

A set of isolated exosomes were PKH67 labelled and added to mesothelial cells to verify that THP-1 cell exosomes interact and are taken up by mesothelial cells. As shown in Figure 5A, green fluorescent labelled exosomes were identified inside mesothelial cells suggesting their uptake by mesothelial cells.

Exosomes from asbestos exposed THP-1 cells cause gene changes in mesothelial cells

Exosomes isolated from asbestos exposed and control THP-1 cells were added to mesothelial cells (HPM3). In this experiment, we also included a positive control by exposing a group of mesothelial cells directly to asbestos fibers. After 96 hours of exposure total RNA was extracted and subjected to microarray analysis. Three groups were labeled as follows: control exosomes (0) (cells exposed to no-asbestos THP-1 exosomes), asbestos exosomes (cells exposed to asbestos exposed THP-1 exosomes) and asbestos fibers (cells directly exposed to asbestos fibers). Cutoff thresholds for analysis were set as anything with a 2 fold or a 1.5 fold transcript-level change with an ANOVA transcript-level p-value less than 0.05.

Our main comparison of interest was the asbestos exosome group versus the control exosome group, whilst vying to draw parallels to the asbestos fiber group to the control exosome group. Our results of 1.5 fold cutoff for the asbestos exosome exposed mesothelial cells compared to control exosome exposed mesothelial cells were that a total of 498 genes

changed significantly, 241 up and 257 down (Figure 5B,C, Table 4). In addition, the comparison between control exosome exposed cells to mesothelial cells directly exposed to asbestos fibers yielded differential expressions of 3,788 genes, 1,803 up and 1,985 down (Figure 5C). Of these two separate comparisons, there were a total of 206 genes that were mutual in their differential expression profiles (Figure 5D). With a more stringent cutoff of 2 fold or more up or down between asbestos exosomes versus control exosomes, we observed a total of 80 significant gene level changes, with 32 up and 48 down regulated. A comparison of gene expression profile in mesothelial cells in response to asbestos exosomes and asbestos fibers only is presented in Table 5.

Six common genes of interest were selected from those genes that were shared in being differentially expressed upon exposure to either asbestos exosomes or asbestos fibers themselves for validation by qRTPCR, three of which were up and three down regulated by asbestos exosomes or asbestos fibers themselves upon addition to mesothelial cells, based on known and potential biological relevance in asbestos exposure. The three chosen which were upregulated were *hCCNB2*, *hEGR1* and *hFANCD2*, and the three downregulated were *hCRELD2*, *hERO1B* and *hJAG1*. Validation by qRTPCR showed the same significant trends as in microarray results in all six genes (Figure 5E). *CCNB2* was chosen because of its significance in regard to MM, although it was not in our list of top 10 over expressed genes.

Discussion

Exposure to asbestos fibers is a major human health concern, as it is causally associated with MM, lung cancer and fibrosis. The scientific and medical communities, at this point,

have yet to delineate a useful set of diagnostic biomarkers for asbestos exposure that may be used to pre-empt the deadly illnesses that result from inhaling the fibers. Due to the fact that such inhalation of asbestos fibers is the primary source of one's exposure, and the first cells to contact the fibers are therefore lung epithelial cells and resident macrophages, our study includes these cell types.

Our study is aimed at exosomal protein abundances in response to asbestos exposure in epithelial cells and macrophages. Additionally, as mesothelial cells are specifically susceptible to asbestos, leading to MM, we wanted to gauge the subsequent effect these exosomes may have on mesothelial cells that could be the targets of such exosomes and thereby beget the development of MM. Our rationale is that it is currently unknown if MM, a tumor arising on the mesothelial cell lining of cavities (i.e. pleura or peritoneum), is the result of direct contact with asbestos fibers migrating from within the lung to the outer lining or from secreted factors (loaded in exosomes) from the original cells to contact the fibers being sent to the mesothelial cells leading to transformation, or perhaps both.

The design herein was to isolate exosomes from asbestos exposed epithelial cells or macrophages, quarry them for proteomic signatures of asbestos exposure, and to also take these purified exosomes and add them to healthy mesothelial cells and analyze for gene expression changes that may be involved in MM tumorigenic process.

The study described in this paper is new, in that we are the first to report on the signature, and potential role, of exosomes in the context of asbestos exposure.

The results of our proteomics analyses of exosomes from asbestos exposed epithelial cells indicate that there is clearly a shift in protein abundances in epithelial exosomes upon

asbestos exposure. We observed increased abundance of thrombospondin-1 in both analyses (mass spectrometry experiment and immunoblot blot validation) of exosomal proteins from asbestos exposed epithelial cells. This is interesting as thrombospondin-1 has been identified as being significantly overexpressed in MM tumors (24). We were also intrigued to see that proteomics analysis indicated higher exosomal abundances of vimentin upon producer cell exposure to asbestos because vimentin is a key regulator in the response to asbestos exposure by regulating the NLRP3 inflammasome and is used as a mesenchymal marker in the transition of mesothelial cells to a more neoplastic state (23, 25). Additionally, we validated the increased exosomal abundance of glypican-1 in exosomes from asbestos exposed cells, which piqued our interest because of its established role as being an exosomal indicator of cancer, most notably as a pancreatic cancer exosome biomarker (12, 26).

Next we studied the effect of epithelial cell exosomes on mesothelial cells. To begin, we confirmed the uptake of said exosomes by using an established method of PKH67 labelling of exosomes and adding to mesothelial cells for visualization of uptake (27, 28). Subsequently, the effect of exosomes from producer epithelial cells (either asbestos exposed or control) was studied on mesothelial cell transformation genes (EMT pathway). Our rationale for this EMT array was because we have shown recently that asbestos exposure causes mesothelial to fibroblastic transition (MFT/EMT) *in vitro* and *in vivo* (23) . Furthermore, many of the known genetic alterations that occur in mesothelial cells that are hallmarks of tumorigenesis and MM are categorized as EMT genes, either the loss of epithelial-like gene expression or gain of more mesenchymal gene expression (29-31).

Our findings were modest changes in multiple EMT genes consistent with the expectation that exosomes from asbestos exposed epithelial cells can lead to changes in mesothelial cells similar to those that would occur if the mesothelial cell was in direct contact with asbestos fibers or undergoing transition to a more mesenchymal state. Our PCR array indicated a significant upregulation in STEAP1 when mesothelial cells were targeted with asbestos exosomes, and increased STEAP1 has been reported as a result of mesothelial cell contact with asbestos fibers (32). We also were encouraged to see that asbestos exosomes lead to marked reduction in IL-1RN, and significant reduction in the expression of known epithelial markers E-cadherin and desmoplakin. Reduction in E-cadherin and desmoplakin expression are well-described as markers for EMT (33, 34), and these alterations have been described in mesothelial cell exposure to asbestos (35, 36). Our conclusions from these epithelial cell experiments are that there is undoubtedly a signature abundance modification in exosomal proteins from epithelial cells exposed to asbestos fibers and that these exosomes are capable to interact with, and alter gene signature in mesothelial cells. Lack of significance in some results could be attributed to either shorter duration of exposure with exosomes or lower concentration of exosome being available to cells.

Next, we studied the macrophages and their influence on mesothelial cells as these are the first cell type to interact with asbestos fibers in the lung along with epithelial cells. Due to previous reports in literature that THPs need priming before responding to stimulus, we performed experiments with and without priming of THPs with PMA before exposing to asbestos. Our proteomic data showed that priming was unnecessary for macrophage

exosomes to be affected by asbestos exposure, therefore, that data will not be enumerated upon.

Our results indicated that exposure to asbestos does indeed alter the abundances of certain exosomal proteins in THP-1 cells. We were primarily interested in only those proteins that were increased upon exposure to asbestos, as our quest is to surmount data that may lead to biomarker discovery. Those proteins of interest increased in asbestos exosomes from macrophages included vimentin (also shown increased in the epithelial study), superoxide dismutase, annexin 5 and we also identified thrombospondin in macrophage exosomes. Vimentin was of interest due to its role in inflammasome initiation and asbestos exposure as listed above. Superoxide dismutase was particularly interesting because of its ability to scavenge oxidants and the fact that it is elevated in asbestos exposure models described elsewhere and in mesothelioma studies (37-40). Our results followed by subsequent validation indicate that exposure to asbestos does lead to protein abundance differences in exosomes from macrophages.

Our next endeavor was to classify if asbestos exosomes from macrophages have an ability to elicit gene expression changes in mesothelial cells. First, we made sure that exosomes were taken up by mesothelial cells by adding PKH67 labeled exosomes to mesothelial cells and visualizing their uptake.

Microarray data analysis showed that asbestos exosomes exposure to mesothelial cells significantly changed the expression of 498 genes compared to cells exposed to control exosomes. Furthermore, as expected and published before (18), direct exposure of mesothelial cells to asbestos fibers altered expression of 3,788 genes, and of these, 206

genes were commonly differentially expressed by both experimental groups compared to control. The fact that we see such gene expression similarities indicates that exosomes from asbestos exposed macrophages undoubtedly have the ability to elicit gene expression changes in mesothelial cells in parallel modes to direct asbestos exposure. Furthermore, finding the gene-changes that were not common with direct asbestos exposure is intriguing as it suggests the capability of exosome contents to affect mesothelial cell gene expression and requires further validation.

To confirm the robustness of our data we validated six genes of interest *EGR1*, *CCNB2*, *FANCD2*, *CRELD2*, *ERO1B*, and *JAG1*. *EGR1* is a transcriptional regulator of genes required for cellular differentiation and mitogenesis, and has been shown by our group to be increased in cells exposed to asbestos (15) and its involvement in mesothelial cell response signaling to asbestos (41). Additionally, we were interested in the up-regulated gene *CCNB2*, a key regulator in cell-cycle machinery, is involved in TGFβ meditated cell-cycle control, and is involved in the instability of chromosomes with its overexpression modifying chromosome segregation and spindle checkpoint (42). Notably, overexpression of *CCNB2* is an attribute of MM (43, 44). Also overexpressed in mesothelial cells exposed to asbestos exosomes from macrophages was the regulator of chromosomal stability, *FANCD2*, which is upregulated in MM, caused by asbestos exposure (45, 46).

As for the observed down-regulated mesothelial cell genes, our interest was the endoplasmic reticulum (ER) stress inducible gene *CRELD2* and the oxidoreductase *ERO1B* involved in ER stress, as asbestos is known to lead to ER stress (47, 48). Lastly, we drew

attention to *JAG1* expression, the notch ligand, which is involved in transcriptional regulation in cancer (49).

Taken together, our data on asbestos exosomes from macrophages provides robust indication for their ability to mirror many of the gene expression changes of mesothelial cells exposed directly to asbestos fibers. This is another addition to the building evidence of the biological importance of exosomes in disease, and supports our hypothesis that exosomes may be the carrier of information from asbestos exposed macrophages to mesothelial cells to cause oncogenic changes and MM. Our report is the first to provide insight on the role of exosomes in asbestos induced mesothelial cell diseases.

This study provided clues to a proteomic signature of exosomes from asbestos exposed epithelial cells and macrophages, that is an initial motion to future exosomal biomarker studies in human subjects exposed to asbestos. We are excited to contribute this data to the field of exosomes and asbestos research as there is undeniable evidence that asbestos exosomes are information conduits that alter gene expression in target mesothelial cells. That exosome induced alteration is remarkably comparable to those changes prompted by direct asbestos fiber contact on mesothelial cells, strongly suggesting that exosomes may be a pivotal player in the human response to asbestos exposure that leads to disease development. Undoubtedly, there are limitations to this study, like the lack of *in vivo* data and the role of inflammasomes and reactive oxygen species on exosome packaging and secretion. We intend to further develop and validate upon this story in future *in vivo* experiments and human serum samples from asbestos exposed individuals. Our future

studies will confirm and finely delineate the role of exosomes in asbestos exposure biology and as mines for biomarker discovery.

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Author Contributions

A. Shukla conceived the idea, P. Munson and A. Shukla designed research; P. Munson and Y. Lam performed research; P. Munson, Y. Lam, and J. Dragon analyzed data; A. Shukla and P. Munson wrote paper; M. MacPherson prepared figures and tables and provided technical help with experiments. All authors reviewed manuscript.

Figures and Figure Legends

Figure 1

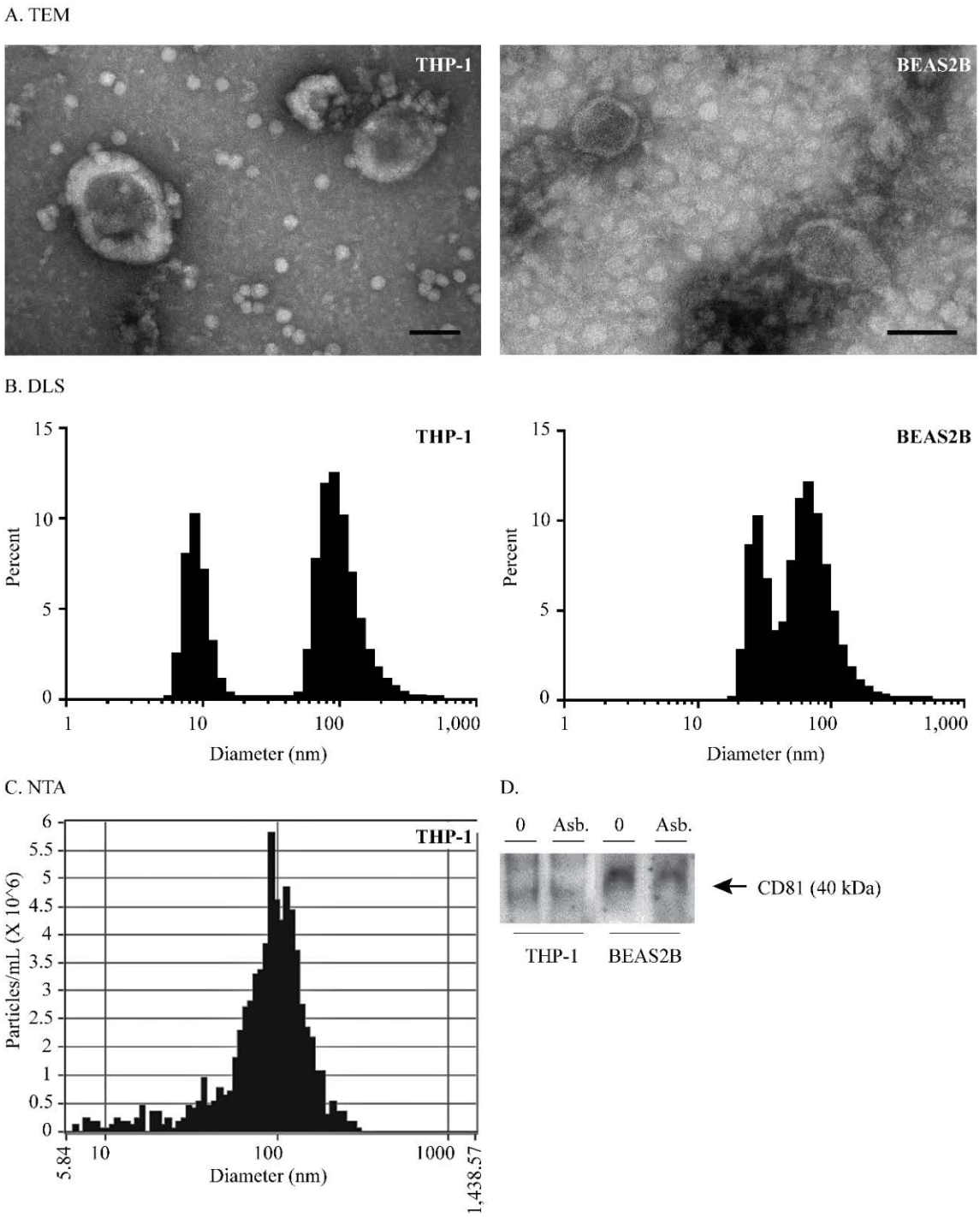

Figure 1. Exosome isolation and characterization from human bronchial epithelial (BEAS2B) cells and THP-1 macrophages. A) TEM showing exosomes membrane bound structure and proper size range (scale bar = 100 nm), B) DLS indicating exosome size distribution, C) NTA indicating exosome size distribution and concentration of particles, and D) Western blot analysis for presence of exosome marker CD81.

Figure 2

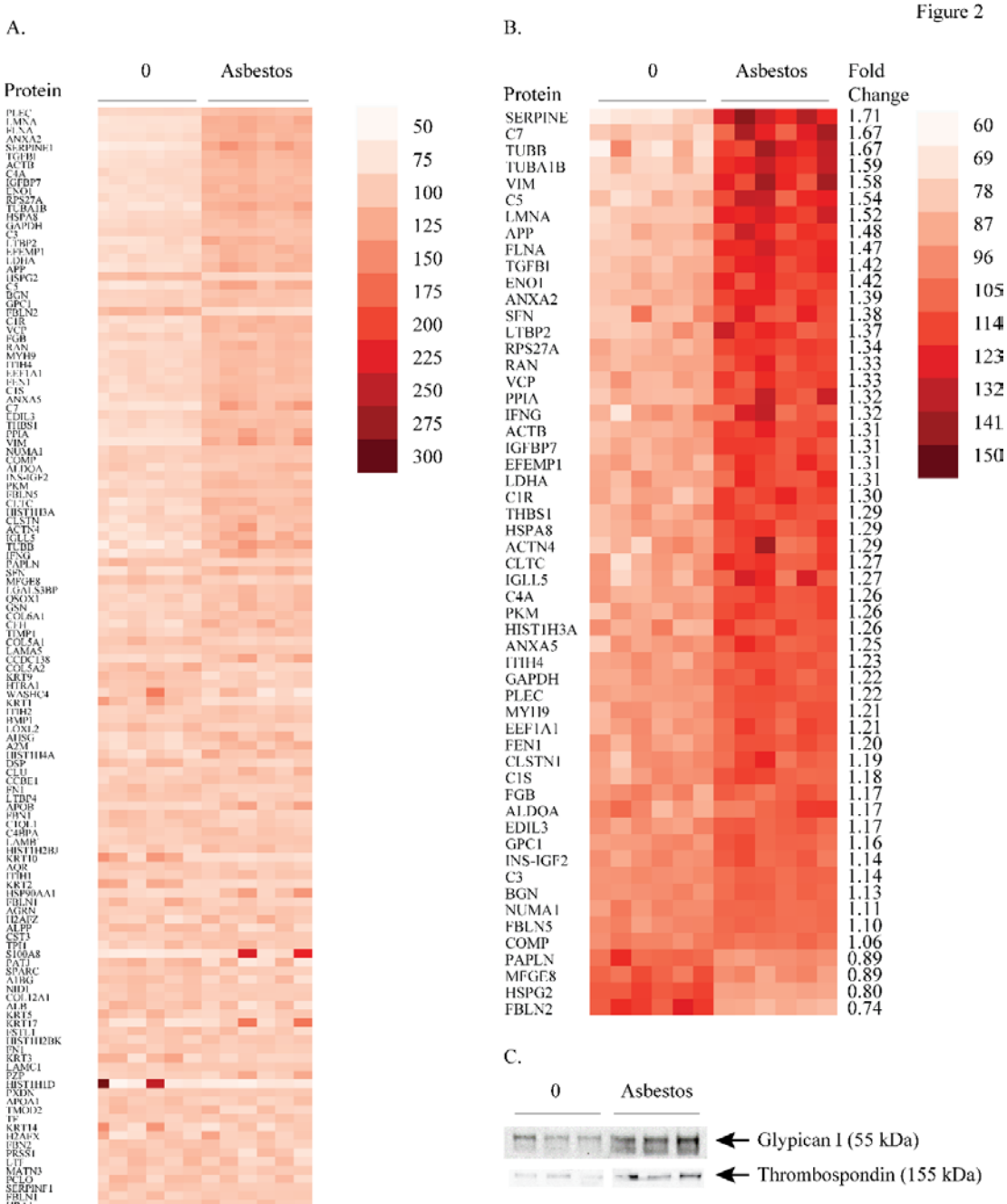
A.
0
Asbestos
Protein
50
75
100
125
150
175
200
225
250
275
300
B.
0
Asbestos
Protein
Fold Change
SERPINE 1.71
C7 1.67
TUBB 1.67
TUBA1B 1.59
VIM 1.58
C5 1.54
LMNA 1.52
APP 1.48
FLNA 1.47
TGFBI 1.42
ENO1 1.42
ANXA2 1.39
SFN 1.38
LTBP2 1.37
RPS27A 1.34
RAN 1.33
VCP 1.33
PPIA 1.32
IFNG 1.32
ACTB 1.31
IGFBP7 1.31
EFEMP1 1.31
LDHA 1.31
C1R 1.30
THBS1 1.29
HSPA8 1.29
ACTN4 1.29
CLTC 1.27
IGLL5 1.27
C4A 1.26
PKM 1.26
HIST1H3A 1.26
ANXA5 1.25
ITIH4 1.23
GAPDH 1.22
PLEC 1.22
MYH9 1.21
EEF1A1 1.21
FEN1 1.20
CLSTN1 1.19
C1S 1.18
FGB 1.17
ALDOA 1.17
EDIL3 1.17
GPC1 1.16
INS-IGF2 1.14
C3 1.14
BGN 1.13
NUMA1 1.11
FBLN5 1.10
COMP 1.06
PAPLN 0.89
MFGE8 0.89
HSPG2 0.80
FBLN2 0.74
60
69
78
87
96
105
114
123
132
141
150
C.
0
Asbestos
Glypican 1 (55 kDa)
Thrombospondin (155 kDa)

Figure 2. Proteomic analysis of exosomes from asbestos exposed and control BEAS2B cells showed different protein signature. A) Heat map indicating the abundances of all proteins identified in both groups, B) expanded heat map section showing all differentially abundant proteins with p-value less than 0.05 (one TMT experiment: asbestos vs. control, 3 biological replicates, 2 technical replicates), listed according to fold change, C) Validation immunoblot of exosomal proteins glypican-1 and thrombospondin.

Figure 3

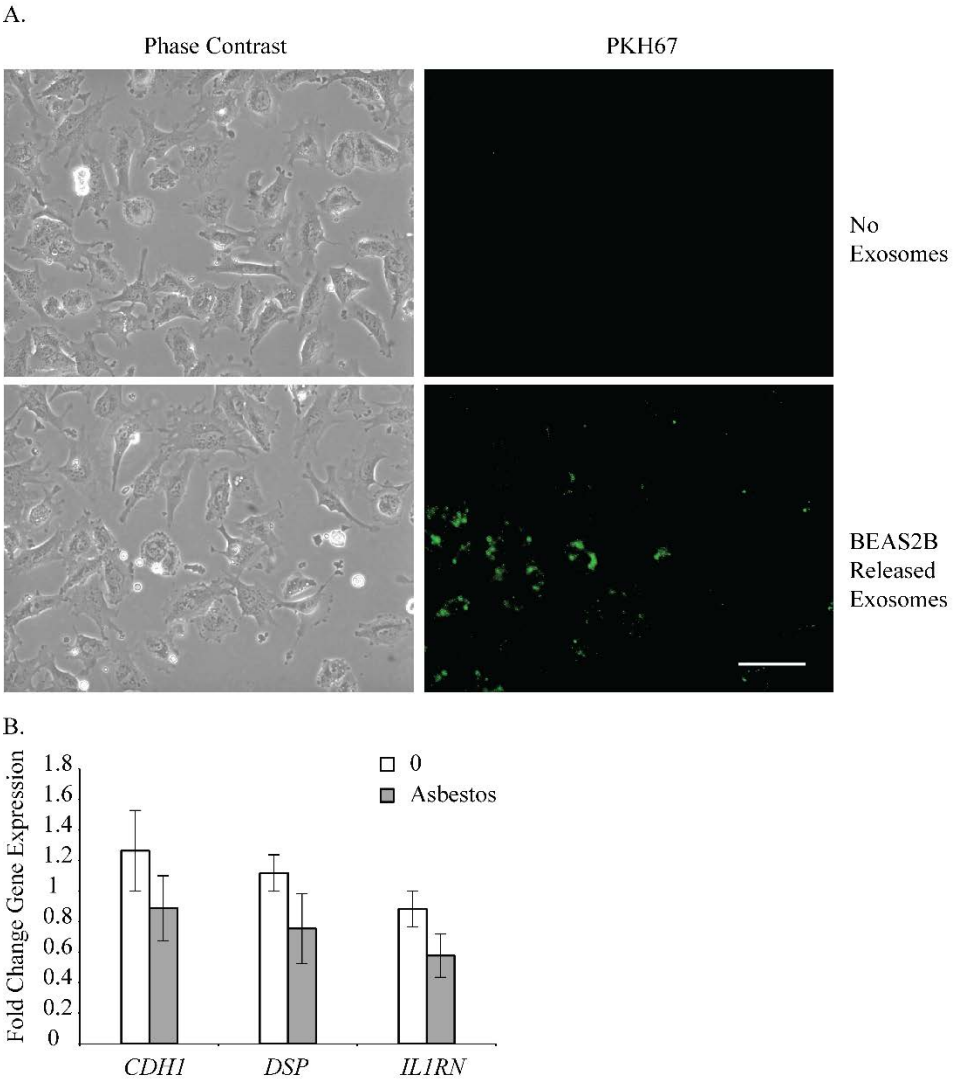

Figure 3. Exosomes from asbestos exposed epithelial cells are taken up by mesothelial cells and caused altered gene expression. A) PKH67 labeled exosomes from BEAS2B cells interact with and are taken up by target mesothelial cells, scale bar = 100 nm, B) qPCR validation of differentially expressed genes (CDH1, DSP, and IL1RN) from PCR Array of mesothelial cells after exposure of cells to exosomes from asbestos exposed BEAS2B cells. n=3/group, statistically not significant by two-tailed t-test.

Figure 4

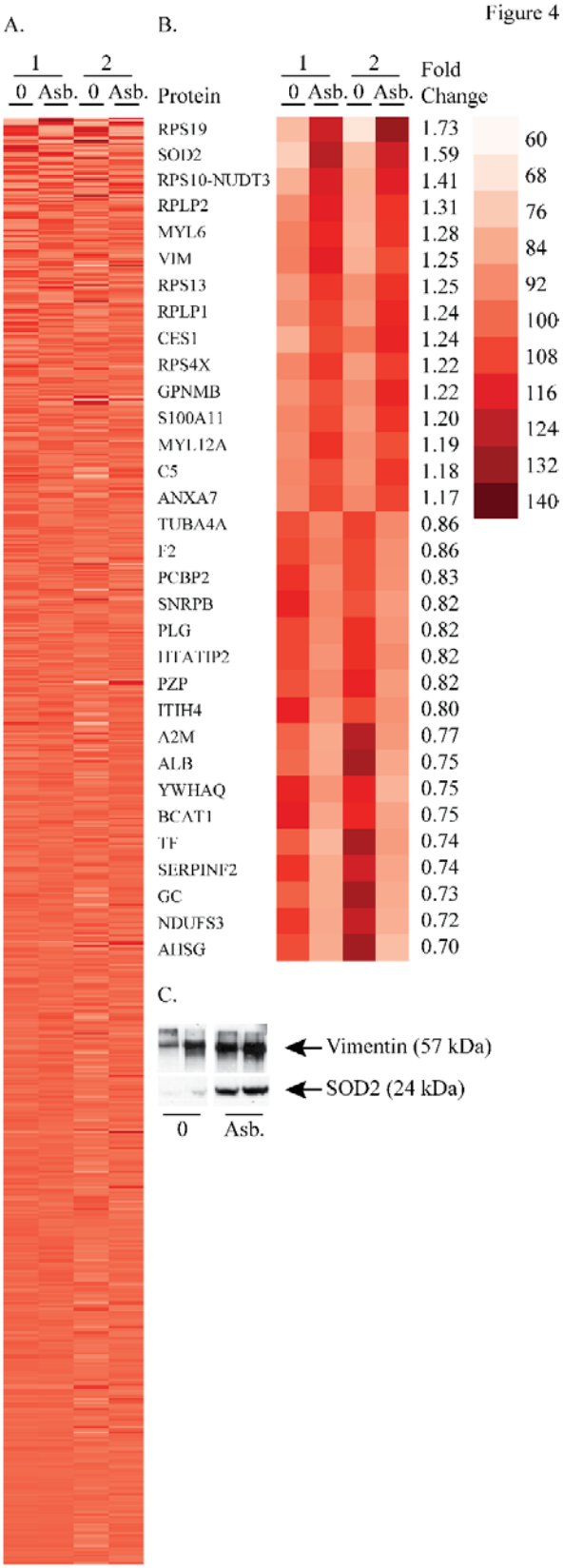
A.
B.
1
2
0 Asb. 0 Asb.
Protein
Fold Change
RPS19 1.73
SOD2 1.59
RPS10-NUDT3 1.41
RPLP2 1.31
MYL6 1.28
VIM 1.25
RPS13 1.25
RPLP1 1.24
CES1 1.24
RPS4X 1.22
GPNMB 1.22
S100A11 1.20
MYL12A 1.19
C5 1.18
ANXA7 1.17
TUBA4A 0.86
F2 0.86
PCBP2 0.83
SNRPB 0.82
PLG 0.82
ITTATIP2 0.82
PZP 0.82
ITIH4 0.80
A2M 0.77
ALB 0.75
YWHAQ 0.75
BCAT1 0.75
TF 0.74
SERPINF2 0.74
GC 0.73
NDUFS3 0.72
AHSG 0.70
60
68
76
84
92
100
108
116
124
132
140
C.
Vimentin (57 kDa)
SOD2 (24 kDa)
0
Asb.

Figure 4. Proteomic analysis showed proteins with differential abundances in asbestos exposed exosomes from THP-1. A) Heat map indicating the abundances of all proteins identified in both groups, sorted according to p-value, (1, 2: two separate TMT experiments) B) expanded heat map region showing exosomal proteins with differential abundance between control group and asbestos exposed group with p-value less than 0.05 (asbestos vs. control, two biological replicates), listed according to fold change, C) Validation immunoblot of exosomal proteins vimentin and SOD2.

Figure 5

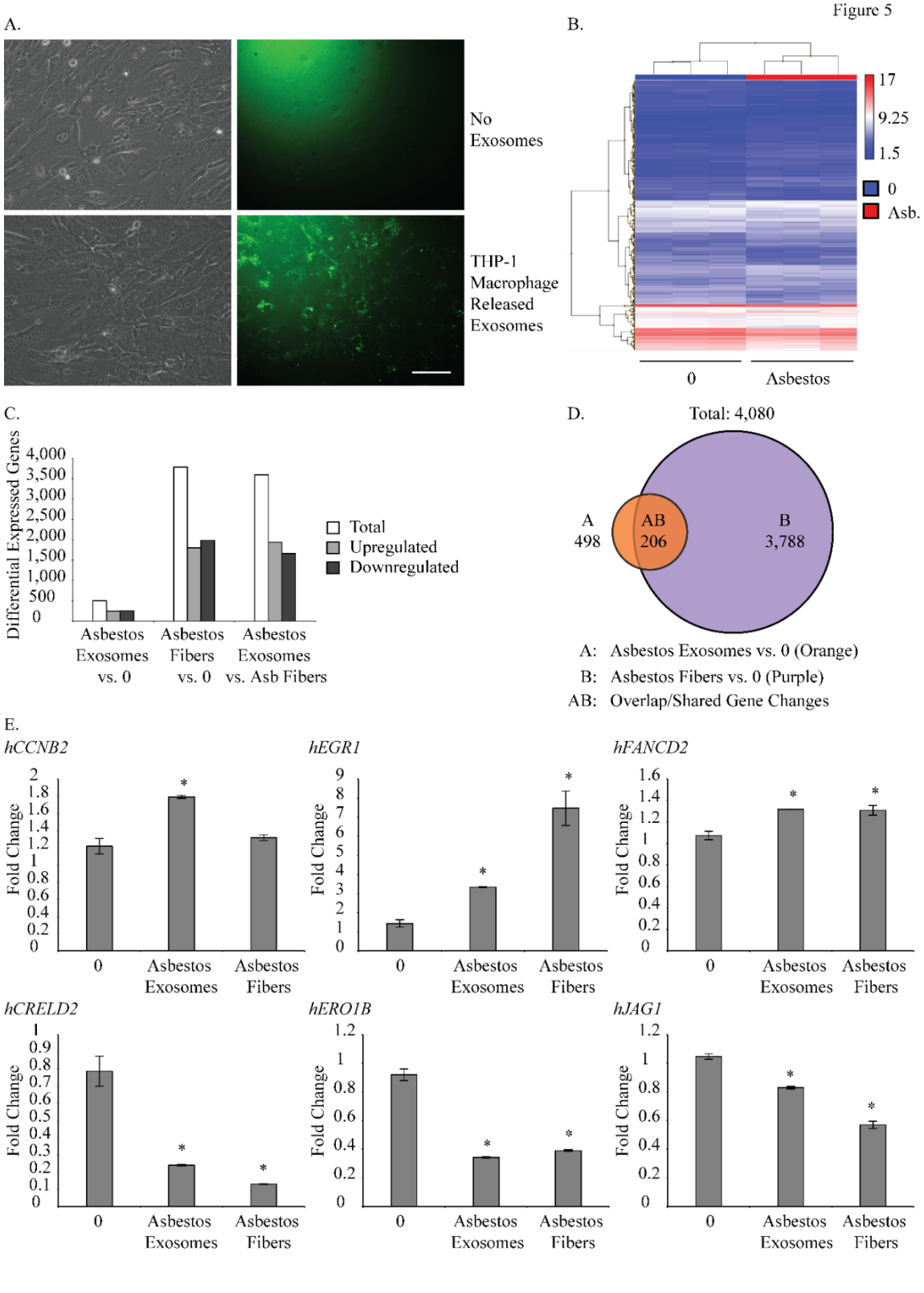
A.
No Exosomes
THP-1 Macrophage Released Exosomes
B.
17
9.25
1.5
0
Asb.
0
Asbestos
C.
Differential Expressed Genes
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
Total
Upregulated
Downregulated
Asbestos Exosomes vs. 0
Asbestos Fibers vs. 0
Asbestos Exosomes vs. Asb Fibers
D.
Total: 4,080
A
498
AB
206
B
3,788
A: Asbestos Exosomes vs. 0 (Orange)
B: Asbestos Fibers vs. 0 (Purple)
AB: Overlap/Shared Gene Changes
E.
hCCNB2
hEGR1
hFANCD2
hCRELD2
hERO1B
hJAG1
Fold Change
0
Asbestos Exosomes
Asbestos Fibers

Figure 5. Exosomes from asbestos exposed THP-1 cells are taken up by mesothelial cells and caused gene expression changes. A) PKH67 labeled exosomes from THP-1 cells interact with and are taken up by target mesothelial cells, scale bar = 100 nm, B) Clariom S microarray heat map of gene expression between control mesothelial cells and mesothelial cells exposed to exosomes from asbestos exposed macrophages, C) Number of differentially expressed genes from microarray analysis in groups of asbestos exosomes vs. control, asbestos fibers vs control, and asbestos exosomes vs. asbestos fibers, D) Venn diagram showing genes differentially expressed between control mesothelial cells and asbestos exosome exposed cells (A), control and asbestos fiber exposed mesothelial cells (B), and the shared genes differentially expressed between both comparisons (AB), E) qPCR validation of genes upregulated in asbestos exosome and asbestos fiber groups compared to control (*CCNB2*, *EGR1*, and *FANCD2*) and genes downregulated in asbestos exosome and asbestos fiber groups compared to control (*CRELD2*, *ERO1B*, and *JAG1*). * indicates p-value ≤ 0.05 by one-way ANOVA.

Table 1: Top upregulated proteins in exosomes collected from asbestos exposed BEAS2B cells as compared to exosomes collected from control cells

Accession	Description	Abundance Ratio	p-Value
P05121	Plasminogen activator inhibitor 1	1.706	0.000490422
P08670	Vimentin	1.582	0.016013921
P21333	Filamin-A	1.466	0.000388539
Q15582	Transforming growth factor-beta-induced protein ig-h3	1.419	0.000522876
P55072	Transitional endoplasmic reticulum ATPase	1.333	0.005639401
P01579	Interferon gamma	1.315	0.033787902
Q16270	Insulin-like growth factor-binding protein 7	1.308	0.000727588
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	1.306	0.002498946
P07996	Thrombospondin-1	1.292	0.01433131
P0C0L4	Complement C4-A	1.263	0.000724361
P08758	Annexin A5	1.248	0.012712613
Q15149	Plectin	1.215	1.41242E-05
P06396	Gelsolin	1.188	0.059612729
P02675	Fibrinogen beta chain	1.17	0.005784599

P35052	Glypican-1	1.163	0.004177749

Table 2: PCR Array analysis showing top up and downregulated genes in HPM3 cells exposed to asbestos administered BEAS2B exosomes

Gene Name	Fold Change	p-Value*
Upregulated		
Six transmembrane epithelial antigen of the prostate 1	**1.298**	**0.018268**
Zinc finger E-box binding homeobox 1	1.2646	0.725035
Versican	1.198	0.153794
Vacuolar protein sorting 13 homolog A (S. cerevisiae)	1.1452	0.139756
Calcium/calmodulin-dependent protein kinase II inhibitor 1	1.1322	0.291454
Notch 1	1.1207	0.604015
Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	1.1198	0.316425
Tetraspanin 13	1.1198	0.197028
PTK2 protein tyrosine kinase 2	1.1171	0.150141
Pleckstrin 2	1.1126	0.188704
PPPDE peptidase domain containing 2	1.1097	0.113347

Protein tyrosine phosphatase type IVA, member 1	1.108	0.334835
Cadherin 2, type 1, N-cadherin (neuronal)	1.1015	0.298931
Downregulated		
Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	0.5824	0.305409
Fibroblast growth factor binding protein 1	0.6299	0.067967
Cadherin 1, type 1, E-cadherin (epithelial)	**0.6597**	**0.048589**
Desmocollin 2	0.7248	0.103433
Interleukin 1 receptor antagonist	**0.7297**	**0.325295**
Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	0.746	0.074611
Desmoplakin	**0.7474**	**0.008235**
SRY (sex determining region Y)-box 10	0.7577	0.09832
Bone morphogenetic protein 7	0.7594	0.102055
Goosecoid homeobox	0.7594	0.102055
Wingless-type MMTV integration site family, member 11	0.7594	0.102055
Human Genomic DNA Contamination	0.7594	0.102055
Positive PCR Control	0.7644	0.128955
Reverse Transcription Control	**0.7786**	**0.048837**
Nodal homolog (mouse)	0.7859	0.12515

*Bold entries, p≤0.05.

Table 3: Top upregulated proteins in exosomes collected from THP-1 cells exposed to asbestos with or without PMA*

Protein	Control	PMA	Asb	PMA+Asb
Vimentin	1	1.1637	2.8122	1.2380
40S ribosomal protein S19	1	0.9677	2.1622	1.9157
Superoxide dismutase [Mn], mitochondrial (Fragment)	1	0.7706	1.7601	1.3151
Isoform 4 of Superoxide dismutase [Mn], mitochondrial	1	0.9338	1.6439	1.3213
40S ribosomal protein S10	1	0.8829	1.6290	1.4406
Myosin light polypeptide 6 (Fragment)	1	1.0701	1.4684	1.2477
Apolipoprotein B-100	1	1.0271	1.4000	0.9767
Glutamine synthetase	1	1.1757	1.3199	1.1531
Isoform 3 of Liver carboxylesterase 1	1	0.8012	1.2963	1.0236
Phosphomevalonate kinase	1	1.3831	1.2602	1.1758
Nuclear mitotic apparatus protein 1 (Fragment)	1	1.5058	1.2517	1.0953
10 kDa heat shock protein, mitochondrial	1	0.8768	1.2365	0.9184

Myosin regulatory light chain 12A	1	1.1347	1.2262	1.2004
Importin-4	1	1.1950	1.2223	1.1424
Actin-related protein 2/3 complex subunit 4	1	0.9399	1.2216	0.9938

*Values are average fold changes relative to control.

Table 4: Microarray analysis showing top up and downregulated genes in HPM3 cells exposed to asbestos administered THP-1 exosomes as compared to control exosomes

Gene Name	Fold Change	p-Value
Upregulated		
Early growth response 1	3.43	0.0096
Meiosis-specific nuclear structural 1	3.22	0.0031
Histone cluster 1, H3g	2.82	0.00003
Transcript Identified by AceView, Entrez Gene ID(s) 4731	2.81	0.001
POTE ankyrin domain family, member C	2.69	0.0034
Long intergenic non-protein coding RNA 663	2.54	0.0075
PARP1 binding protein	2.53	0.009
Fanconi anemia complementation group D2	2.47	0.024
Transcript Identified by AceView, Entrez Gene ID(s) 79677	2.42	0.0148
Chromosome 16 open reading frame 52	2.34	0.0402
Downregulated		

Cysteine rich with EGF-like domains 2	0.156	0.0013
Glycoprotein Ib (platelet), beta polypeptide; septin 5	0.225	0.0178
Stromal cell-derived factor 2-like 1	0.271	0.0073
Endoplasmic reticulum oxidoreductase beta	0.285	0.0005
Schlafen family member 11	0.314	0.005
Jagged 1	0.321	0.0002
Arginase 2	0.344	0.0325
Cell division cycle 6	0.351	0.0091
Transcript Identified by AceView, Entrez Gene ID(s) 153339	0.365	0.0188
GTPase, IMAP family member 2	0.373	0.0158

References

1. Berman, D. W., and Crump, K. S. (2008) Update of potency factors for asbestos-related lung cancer and mesothelioma. *Critical reviews in toxicology* **38 Suppl 1**, 1-47
2. Markowitz, S. B., Levin, S. M., Miller, A., and Morabia, A. (2013) Asbestos, asbestosis, smoking, and lung cancer. New findings from the North American insulator cohort. *American journal of respiratory and critical care medicine* **188**, 90-96
3. IARC, W. (2012) Asbestos (chrysotile, amosite, crocidolite, tremolite, actinolite, and anthophyllite). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans A Review of Human Carcinogens;* **100C**, 219-309
4. Frank, A. L., and Joshi, T. K. (2014) The global spread of asbestos. *Annals of global health* **80**, 257-262
5. Mossman, B. T., and Churg, A. (1998) Mechanisms in the pathogenesis of asbestosis and silicosis. *American journal of respiratory and critical care medicine* **157**, 1666-1680
6. Dostert, C., Petrilli, V., Van Bruggen, R., Steele, C., Mossman, B. T., and Tschopp, J. (2008) Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science (New York, N.Y.)* **320**, 674-677
7. Li, M., Gunter, M. E., and Fukagawa, N. K. (2012) Differential activation of the inflammasome in THP-1 cells exposed to chrysotile asbestos and Libby "six-mix" amphiboles and subsequent activation of BEAS-2B cells. *Cytokine* **60**, 718-730
8. Sen, D. (2015) Working with asbestos and the possible health risks. *Occupational medicine (Oxford, England)* **65**, 6-14
9. Raposo, G., and Stoorvogel, W. (2013) Extracellular vesicles: exosomes, microvesicles, and friends. *The Journal of cell biology* **200**, 373-383
10. Andaloussi, E. L., Mager, I., Breakefield, X. O., and Wood, M. J. (2013) Extracellular vesicles: biology and emerging therapeutic opportunities. *Nature reviews. Drug discovery* **12**, 347-357
11. Peterson, M. F., Otoc, N., Sethi, J. K., Gupta, A., and Antes, T. J. Integrated systems for exosome investigation. *Methods (San Diego, Calif.)*
12. Melo, S. A., Luecke, L. B., Kahlert, C., Fernandez, A. F., Gammon, S. T., Kaye, J., LeBleu, V. S., Mittendorf, E. A., Weitz, J., Rahbari, N., Reissfelder, C., Pilarsky, C., Fraga, M. F., Piwnica-Worms, D., and Kalluri, R. (2015) Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* **523**, 177-182
13. Hegmans, J. P., Bard, M. P., Hemmes, A., Luider, T. M., Kleijmeer, M. J., Prins, J. B., Zitvogel, L., Burgers, S. A., Hoogsteden, H. C., and Lambrecht, B. N. (2004) Proteomic analysis of exosomes secreted by human mesothelioma cells. *The American journal of pathology* **164**, 1807-1815
14. Greening, D. W., Ji, H., Chen, M., Robinson, B. W., Dick, I. M., Creaney, J., and Simpson, R. J. (2016) Secreted primary human malignant mesothelioma exosome signature reflects oncogenic cargo. *Scientific reports* **6**, 32643
15. Perkins, T. N., Peeters, P. M., Shukla, A., Arijs, I., Dragon, J., Wouters, E. F. M., Reynaert, N. L., and Mossman, B. T. (2015) Indications for distinct pathogenic mechanisms of asbestos and silica through gene expression profiling of the response of lung epithelial cells. *Human molecular genetics* **24**, 1374-1389

16. Thompson, J. K., Westbom, C. M., MacPherson, M. B., Mossman, B. T., Heintz, N. H., Spiess, P., and Shukla, A. (2014) Asbestos modulates thioredoxin-thioredoxin interacting protein interaction to regulate inflammasome activation. *Part Fibre Toxicol* **11**, 24
17. Shukla, A., MacPherson, M. B., Hillegass, J., Ramos-Nino, M. E., Alexeeva, V., Vacek, P. M., Bond, J. P., Pass, H. I., Steele, C., and Mossman, B. T. (2009) Alterations in gene expression in human mesothelial cells correlate with mineral pathogenicity. *American journal of respiratory cell and molecular biology* **41**, 114-123
18. Dragon, J., Thompson, J., MacPherson, M., and Shukla, A. (2015) Differential Susceptibility of Human Pleural and Peritoneal Mesothelial Cells to Asbestos Exposure. *Journal of cellular biochemistry* **116**, 1540-1552
19. Hillegass, J. M., Miller, J. M., MacPherson, M. B., Westbom, C. M., Sayan, M., Thompson, J. K., Macura, S. L., Perkins, T. N., Beuschel, S. L., Alexeeva, V., Pass, H. I., Steele, C., Mossman, B. T., and Shukla, A. (2013) Asbestos and erionite prime and activate the NLRP3 inflammasome that stimulates autocrine cytokine release in human mesothelial cells. *Part Fibre Toxicol* **10**, 39
20. Eitan, E., Green, J., Bodogai, M., Mode, N. A., Baek, R., Jorgensen, M. M., Freeman, D. W., Witwer, K. W., Zonderman, A. B., Biragyn, A., Mattson, M. P., Noren Hooten, N., and Evans, M. K. (2017) Age-Related Changes in Plasma Extracellular Vesicle Characteristics and Internalization by Leukocytes. *Scientific reports* **7**, 1342
21. Helwa, I., Cai, J., Drewry, M. D., Zimmerman, A., Dinkins, M. B., Khaled, M. L., Seremwe, M., Dismuke, W. M., Bieberich, E., Stamer, W. D., Hamrick, M. W., and Liu, Y. (2017) A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PloS one* **12**, e0170628
22. Hristova, M., Veith, C., Habibovic, A., Lam, Y. W., Deng, B., Geiszt, M., Janssen-Heininger, Y. M., and van der Vliet, A. (2014) Identification of DUOX1-dependent redox signaling through protein S-glutathionylation in airway epithelial cells. *Redox biology* **2**, 436-446
23. Thompson, J. K., MacPherson, M. B., Beuschel, S. L., and Shukla, A. (2017) Asbestos-Induced Mesothelial to Fibroblastic Transition Is Modulated by the Inflammasome. *The American journal of pathology* **187**, 665-678
24. Ohta, Y., Shridhar, V., Kalemkerian, G. P., Bright, R. K., Watanabe, Y., and Pass, H. I. (1999) Thrombospondin-1 expression and clinical implications in malignant pleural mesothelioma. *Cancer* **85**, 2570-2576
25. dos Santos, G., Rogel, M. R., Baker, M. A., Troken, J. R., Urich, D., Morales-Nebreda, L., Sennello, J. A., Kutuzov, M. A., Sitikov, A., Davis, J. M., Lam, A. P., Cheresh, P., Kamp, D., Shumaker, D. K., Budinger, G. R., and Ridge, K. M. (2015) Vimentin regulates activation of the NLRP3 inflammasome. *Nature communications* **6**, 6574
26. Lu, H., Niu, F., Liu, F., Gao, J., Sun, Y., and Zhao, X. (2017) Elevated glypican-1 expression is associated with an unfavorable prognosis in pancreatic ductal adenocarcinoma. *Cancer medicine* **6**, 1181-1191
27. Dutta, S., Warshall, C., Bandyopadhyay, C., Dutta, D., and Chandran, B. (2014) Interactions between Exosomes from Breast Cancer Cells and Primary Mammary Epithelial Cells Leads to Generation of Reactive Oxygen Species Which Induce DNA Damage Response, Stabilization of p53 and Autophagy in Epithelial Cells. *PloS one* **9**, e97580
28. Montecalvo, A., Shufesky, W. J., Stolz, D. B., Sullivan, M. G., Wang, Z., Divito, S. J., Papworth, G. D., Watkins, S. C., Robbins, P. D., Larregina, A. T., and Morelli, A. E. (2008)

Exosomes as a short-range mechanism to spread alloantigen between dendritic cells during T cell allorecognition. *Journal of immunology (Baltimore, Md. : 1950)* **180**, 3081-3090

29. Schramm, A., Opitz, I., Thies, S., Seifert, B., Moch, H., Weder, W., and Soltermann, A. (2010) Prognostic significance of epithelial-mesenchymal transition in malignant pleural mesothelioma. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* **37**, 566-572
30. Casarsa, C., Bassani, N., Ambrogi, F., Zabucchi, G., Boracchi, P., Biganzoli, E., and Coradini, D. (2011) Epithelial-to-mesenchymal transition, cell polarity and stemness-associated features in malignant pleural mesothelioma. *Cancer letters* **302**, 136-143
31. Fassina, A., Cappellesso, R., Guzzardo, V., Dalla Via, L., Piccolo, S., Ventura, L., and Fassan, M. (2012) Epithelial-mesenchymal transition in malignant mesothelioma. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **25**, 86-99
32. Thompson, J. K., MacPherson, M. B., Beuschel, S. L., and Shukla, A. Asbestos-Induced Mesothelial to Fibroblastic Transition Is Modulated by the Inflammasome. *The American journal of pathology* **187**, 665-678
33. Lamouille, S., Xu, J., and Derynck, R. (2014) Molecular mechanisms of epithelial–mesenchymal transition. *Nature reviews. Molecular cell biology* **15**, 178-196
34. Kalluri, R., and Weinberg, R. A. (2009) The basics of epithelial-mesenchymal transition. *The Journal of Clinical Investigation* **119**, 1420-1428
35. Tamminen, J. A., Myllarniemi, M., Hyytiainen, M., Keski-Oja, J., and Koli, K. (2012) Asbestos exposure induces alveolar epithelial cell plasticity through MAPK/Erk signaling. *Journal of cellular biochemistry* **113**, 2234-2247
36. Kim, M. C., Cui, F. J., and Kim, Y. (2013) Hydrogen peroxide promotes epithelial to mesenchymal transition and stemness in human malignant mesothelioma cells. *Asian Pacific journal of cancer prevention : APJCP* **14**, 3625-3630
37. Franko, A., Dodi, #x10D, -Fikfak, M., Arneri, #x107, , N., Dol, #x17E, and an, V. (2009) Manganese and Extracellular Superoxide Dismutase Polymorphisms and Risk for Asbestosis. *Journal of Biomedicine and Biotechnology* **2009**, 6
38. Mossman, B. T., Marsh, J. P., and Shatos, M. A. (1986) Alteration of superoxide dismutase activity in tracheal epithelial cells by asbestos and inhibition of cytotoxicity by antioxidants. *Laboratory investigation; a journal of technical methods and pathology* **54**, 204-212
39. Hirvonen, A., Tuimala, J., Ollikainen, T., Linnainmaa, K., and Kinnula, V. (2002) Manganese superoxide dismutase genotypes and asbestos-associated pulmonary disorders. *Cancer letters* **178**, 71-74
40. Kahlos, K., Pääkkö, P., Kurttila, E., Soini, Y., and Kinnula, V. L. (2000) Manganese superoxide dismutase as a diagnostic marker for malignant pleural mesothelioma. *British journal of cancer* **82**, 1022-1029
41. Shukla, A., Bosenberg, M. W., MacPherson, M. B., Butnor, K. J., Heintz, N. H., Pass, H. I., Carbone, M., Testa, J. R., and Mossman, B. T. (2009) Activated cAMP Response Element Binding Protein Is Overexpressed in Human Mesotheliomas and Inhibits Apoptosis. *The American journal of pathology* **175**, 2197-2206

42. Sarafan-Vasseur, N., Lamy, A., Bourguignon, J., Le Pessot, F., Hieter, P., Sesboue, R., Bastard, C., Frebourg, T., and Flaman, J. M. (2002) Overexpression of B-type cyclins alters chromosomal segregation. *Oncogene* **21**, 2051-2057
43. Bruno, R., Poma, A. M., Alì, G., Giannini, R., Puppo, G., Melfi, F., Lucchi, M., Mussi, A., Falcone, A., Chella, A., and Fontanini, G. (2017) Novel prognostic markers for epithelioid malignant pleural mesothelioma. *Journal of Clinical Oncology* **35**, e20028-e20028
44. Crispi, S., Calogero, R. A., Santini, M., Mellone, P., Vincenzi, B., Citro, G., Vicidomini, G., Fasano, S., Meccariello, R., Cobellis, G., Menegozzo, S., Pierantoni, R., Facciolo, F., Baldi, A., and Menegozzo, M. (2009) Global Gene Expression Profiling Of Human Pleural Mesotheliomas: Identification of Matrix Metalloproteinase 14 (MMP-14) as Potential Tumour Target. *PloS one* **4**, e7016
45. Nabavi, N., Bennewith, K. L., Churg, A., Wang, Y., Collins, C. C., and Mutti, L. (2016) Switching off malignant mesothelioma: exploiting the hypoxic microenvironment. *Genes & cancer* **7**, 340-354
46. Roe, O. D., Anderssen, E., Sandeck, H., Christensen, T., Larsson, E., and Lundgren, S. (2010) Malignant pleural mesothelioma: genome-wide expression patterns reflecting general resistance mechanisms and a proposal of novel targets. *Lung cancer (Amsterdam, Netherlands)* **67**, 57-68
47. Ryan, A. J., Larson-Casey, J. L., He, C., Murthy, S., and Carter, A. B. (2014) Asbestos-induced disruption of calcium homeostasis induces endoplasmic reticulum stress in macrophages. *The Journal of biological chemistry* **289**, 33391-33403
48. Kamp, D. W., Liu, G., Cheresh, P., Kim, S. J., Mueller, A., Lam, A. P., Trejo, H., Williams, D., Tulasiram, S., Baker, M., Ridge, K., Chandel, N. S., and Beri, R. (2013) Asbestos-induced alveolar epithelial cell apoptosis. The role of endoplasmic reticulum stress response. *American journal of respiratory cell and molecular biology* **49**, 892-901
49. Guo, L., Zhang, T., Xiong, Y., and Yang, Y. (2015) Roles of NOTCH1 as a Therapeutic Target and a Biomarker for Lung Cancer: Controversies and Perspectives. *Disease markers* **2015**, 520590

CHAPTER FOUR

Cancer Exosomes to treat the Cancer: Potential Novel Therapeutic Tactics against Mesothelioma

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Abstract

Malignant mesothelioma (MM) is an aggressive cancer of the mesothelial surface of organ cavities, almost exclusively a direct result of asbestos exposure. The disease is essentially incurable with no means early diagnosis and the standard chemotherapeutic regimens do not extend the life of patients. Our group recently began a quest into surveying MM tumor biology with a focus on exosome-contained microRNAs (miRNAs). We discovered that the most abundant miRNAs in MM cancer exosomes were tumor suppressors, particularly the pro-apoptotic miR-16-5p. This observation lead us to hypothesize that MM cells preferentially secreted tumor-suppressor miRNAs via exosomes. Through separate avenues of potential therapeutic advance, we embarked on an innovative strategy to kill MM tumor cells. We inhibited exosome secretion using small molecule inhibitors, thereby down-regulating miR-16-5p in exosomes and rebuilding cellular miR-16-5p leading to loss of proliferation/cell death, decreased migration/invasion, and reduced of miR-16-5p target oncoproteins CCND1 and BCL2. In addition, we force-fed MM tumor exosomes back to the producer MM tumor cells, leading to increased levels of cell death, and a reduction in the same oncoproteins as seen in our exosome inhibition trials. We also recapitulated these results with direct transfection of miR-16-5p.We demonstrated this phenomenon in multiple MM cell lines and confirmed that this is a cancer-cell specific effect. Additionally, we uncovered a mechanism of miR-16-5p loading into exosomes by the RNA binding protein HuR. Our data provide novel evidence on a tumorigenic mechanism of MM tumor cells by preferential secretion of miR-16-5p within exosomes.

Introduction

Malignant mesothelioma (MM) is a remarkably deadly cancer arising after exposure to asbestos fibers (1). Median life-span after diagnosis is 6-12 months, there is a latency period of 20-50 years after initial asbestos exposure, and MM is relatively un-diagnosable until the disease is in advanced stages (2). Due to limited knowledge of biomarkers for asbestos exposure and early detection of this cancer, coupled with no successful therapeutic regimens other than chemotherapeutic intervention with cisplatin and pemetrexed, this disease signifies large gaps in scientific knowledge that, when filled, would greatly benefit human health (3).

An exciting realm of cancer research has developed over the past decade by focusing on nano-sized extracellular vesicles, known as exosomes, to answer pivotal problems such as those mentioned above. Exosomes refer to a class of vesicles produced via the endocytic pathway and ranging in size from 30-140nm in diameter. As a new piece to the puzzle of cancer, exosomes represent an important aspect of biological signaling between cells and as a means of novel biomarker identification strategies (4). This is directly linked to the biofunctional cargo enriched in exosomes such as proteins, miRNAs, and lipids (5,6).

To date there are only a handful of publications focusing on exosomes in the disease setting of mesothelioma. The initial steps towards this area were to analyze the proteomic make up of exosomes isolated from pleural effusions (7) and separately by mesothelioma tumor cells (8). A decade after these reports, it was shown that tumor-derived exosomes could be used in dendritic-cell (DC) based immunotherapeutic strategies against mesothelioma by treating tumor-bearing mice with DCs loaded with MM exosomes, showing that the

exosomes imparted the mice with an immunological response against the MM thereby increasing survival rate (9). There have been multiple of investigations into the miRNAs involved in mesothelioma, particularly by Glen Reid's group who has summarized a large swath of such knowledge and reported miRNA levels in MM tumor cells and tissues. Of note, the study indicated very low expression levels of tumor suppressor miRNAs in MM such as miR-16-5p, miR-15, miR-31, and let-7a, to name a few (10). Notably, only one current research article looks at the miRNA signature associated with circulating extracellular vesicle (EV) miRNAs in MM patients, and found that miR-103a-3p and miR-30e-3p were discriminatory for MM from asbestos-exposed patients with no cancer (11). Recently, our group has shown differential abundance of exosome proteomic signatures in mouse-serum after asbestos exposure (12), and suggested a novel mechanism by which MM may develop by exosomes traveling from asbestos exposed cells to mesothelial cells thereby modifying the mesothelial cells' gene expression patterns (13).

Our present study is the first to present a quarry into the exosomal miRNAs of MM along with findings implicating new avenues of potential biomarkers and therapeutic options. Here, we investigate the signature miRNAs in MM tumor cell exosomes, and formulated a hypothesis that MM tumor cells preferentially secrete the tumor suppressor miR-16-5p via exosomes. Furthermore, we demonstrated that by inhibiting exosome secretion or force-feeding cancer exosomes back to MM cells can rebuild miR-16-5p levels in the cancer cells resulting in significant killing of cancer cells. In addition, we implicated the RNA binding protein, HuR, as being involved in the mechanism of miR-16-5p loading into exosomes. Our findings may lead to potential therapeutic strategies for MM in future.

Materials & Methods

Cell Culture

Human MM cell lines, H2373, H2595, and HP-1 were kindly contributed by Dr. Harvey Pass (New York University, New York, NY) (14) and Hmeso cells were isolated by Reale et al. (15). Human primary pleural mesothelial cells HPM3 and human immortalized peritoneal mesothelial LP9/TERT-1 (LP9) cells were purchased from Brigham and Women's Hospital, Harvard University, Boston, MA.

All cell lines were cultured as previously reported (16). Cell lines were validated by STR DNA fingerprinting using the Promega CELL ID System (Promega, Madison, WI) (16).

Cisplatin was purchased from Alfa Aesar (Ward Hill, MA) and concentrations for the present study were selected based on previously published literature for MM cells (17). GW4869, Cl-amidine (chloramidine), and bisindolylmaleimide-I were purchased from Cayman Chemical (Ann Arbor, MI) and used at concentrations based on published reports indicating successful inhibition of exosome release from cells (18,19). DMSO in equal volume added to control wells as vehicle control.

Immunostaining of MM cells for HuR

Hmeso cells were fixed in 4% PFA, blocked, washed, and incubated overnight at 4°C with HuR antibody (Cell Signaling Technology, Danvers, MA) as previously described (16). For a negative control, one slide was stained as described, excluding primary antibody. After further washing, cells were incubated with a fluorescently conjugated secondary antibody, AlexaFluor® 647 (Thermo Fisher, Grand Island, NY). Following nuclear

staining with DAPI (Thermo Fisher), sections were imaged with a Nikon A1R-ER Confocal Microscope.

Exosome Isolation and Characterization

Exosome Isolation from cell culture medium

Exosomes were isolated using ExoQuick-TC precipitation reagent (System BioSciences, Palo Alto, CA, USA), as previously described (13).

Transmission Electron Microscopy (TEM)

Double membrane structure of exosomes and size was confirmed using transmission electron microscope (JEOL 1400 TEM) as previously published (13).

Nanoparticle Tracking Analysis

Exosomes number and size were further assessed by nanoparticle tracking analysis (NTA) using the ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and Software ZetaView 8.02.31(13).

Characterization of exosomes by Western blot analysis

Two aliquots of isolated exosomes from representative groups were characterized by immunoblot analysis for presence of exosomal marker CD81 (Sigma Aldrich) and also for absence of calnexin (Novus Biologicals, Littleton, CO, USA) to rule out contaminating ER vesicles (13).

MicroRNA Isolation and Microarray

Isolation of miRNA from exosome pellets was accomplished using Qiagen miRNeasy Micro Kit (Venlo, Netherlands) by adding QiaZol reagent directly to pellets and following the manufacturer's protocol.

RNA quality from exosomes was assessed prior to microarray analysis using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and subsequently the RNA was analyzed using GeneChip™ miRNA 4.0 Array (Thermo Fisher Scientific, Waltham, MA, USA) performed on exosomal miRNA from HPM3, Hmeso and H2373 cells (n=2). Data was analyzed using Transcriptome Analysis Console 4.0 (Thermo Fisher). Parameters were set to any gene that was expressed differently by both 2 fold and 1.5 fold up or down with an ANOVA p-value less than 0.05.

Validation of expression changes in selected miRNAs of interest was conducted by qRTPCR after cDNA synthesis from exosomal miRNA (normalized to 2uL exosome miRNA or 2ng miRNA from cells) using TaqMan Advanced cDNA miRNA cDNA Synthesis Kit (Thermo Fisher, Waltham, MA) following the manufacturer's protocol. We used TaqMan Assays on Demand primers and probes for human miRNAs miR-16-5p, miR-30a-5p, miR-222-3p, and miR-31-5p, and for internal control miRNA cel-miR-39-3p (Thermo Fisher) was used.

Exosome secretion inhibition from cells

Exoxome secretion from cells was inhibited by using 2 different small molecule inhibitors described above.

Immunoblot analysis

Cellular proteins of interest (miR-16-5p targets and HuR) were assessed by immunoblot analysis using antibodies specific to CCND1 and BCL-2 (Abcam) or HuR (Cell Signalling) as previously published (16). Proteins selected for immunoblot analysis were of biological relevance as targets of miR-16-5p regulation.

MTS Assay

Cell viability was determined in various experiments by MTS Assay CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) as per the manufacturer's recommendations (20).

In Vitro Tumorigenic Assays

Mesothelioma cells were treated with cisplatin/exosome inhibitors/transfected with miRNA mimics, and were assessed for various tumorigenic assays as described below.

3-D model to grow mesothelioma spheroids

Mesothelioma cells were grown in a 3-D model using the Cultrex 3-D Spheroid Colorimetric Proliferation/Viability Assay from Trevigen, Inc. (Gaithersburg, MD). Mesothelioma cells were seeded at a density of 2,500/well following the manufacturer's protocol. Six days later colorimetric analysis (MTT) was performed as stated in the manufacturer's protocol (20).

Migration Assay

Migration of MM cells was assessed using 6-well Transwell polycarbonate filters (Corning Costar Corp., Corning, NY) with an 8-μm pore size as described previously

Invasion Assay

Invasiveness of MM cells was assessed using 24-well Transwell polycarbonate filters (Corning Costar Corp., Corning, NY) with an 8-μm pore size with 1mg/mL Matrigel coating gel on upper well as described previously (16).

siRNA & miRNA Transfection

On-Target plus Non-Targeting small-interfering RNA (siRNA) (scrambled control) or On-Target plus SMARTpool human ELAV1 (HuR) siRNA (100 nmol/L, Dharmacon, Lafayette, CO) were transfected into 95% confluent cells using Lipofectamine RNAiMAX (Invitrogen), following the manufacturer's protocol. The efficiency of HuR protein knockdown was determined by Western blot analysis after 48hr. Two separate lots of siRNA were used in duplicate for each siHuR experiment.

MISSION miRNA mimic miR-16-5p and MISSION miRNA negative control were transfected into 95% confluent cells using Lipofectamine RNAiMAX (Invitrogen), following the manufacturers protocol. Two lots of miR-16 mimic were used in duplicate for each transfection experiment.

Success of transfection was verified by protein or RNA levels of transfected RNA.

Exosome Uptake by MM Cells

Exosomes were labeled using PKH67 dye (Sigma Aldrich) according to the manufacturer's protocol. Labeled exosomes were suspended in PBS and added to target cells and imaged on an Olympus IX70 inverted light microscope as described previously (13).

Exosome Force Feeding to MM cells

Exosomes were isolated from Hmeso MM cancer cells and equal volumes of exosome preparation rather than protein content from different groups were added to Hmeso cells. After 24 hr of exposure with exosomes, cells were imaged by phase contrast microscopy with 20× objective lens and subsequently analyzed by MTS assay or cell protein lysate was used for immunoblot.

Statistical analysis

All experiments were performed in duplicate or triplicate and repeated at least twice. A one-way analysis of variance (ANOVA) followed by a Newman-Keuls procedure for adjustment of multiple pairwise comparisons or the student's unpaired two-tailed t-test was applied to all data to establish the significance of observed differences between the various experimental groups. $p \leq 0.05$ was considered significant. All statistical analyses were performed using the GraphPad Prism software program version 7.0 (GraphPad Software, La Jolla, CA).

Results

Exosome Isolation and Characterization from MM Cells

The isolation of exosome samples was characterized by transmission electron microscopy (TEM), Western blot analysis for exosomal marker CD81, and nanoparticle tracking analysis (NTA) (Supplementary Figure 1). The vesicle isolates are predominantly in the size range of 30-140nm in diameter as seen in TEM and NTA, and are enriched in CD81. TEM also indicates the well-described "cup-shape" morphology of the exosomes, and none of the exosomal samples showed a signal for calnexin, suggesting no presence of endoplasmic reticulum contaminants.

Mesothelioma Cancer Cells Secrete High Levels of miR-16-5p in Exosomes

miRNA microarray profiling was conducted on isolated exosomal RNA from MM cell lines and primary mesothelial cells to compare non-cancer versus cancer signature (Figure 1A). There were a total of 20 exosomal miRNAs upregulated and 110 downregulated in expression from MM tumor cell exosomes as compared to exosomes from primary

mesothelial cells, with a >2-fold cut-off for both parameters. All 130 miRNAs with >2-fold differential expression are significant with ANOVA p-value < 0.05 (Supplementary Table 1 supplementary data).

We chose to validate the exosomal miRNA expression levels of 3 upregulated (miR-16-5p, miR-222-3p, miR-30a-5p) and one down regulated (miR-31-5p) miRNA by qRTPCR (Figure 1B), all of which have been implicated in MM biology. Additionally, we validated exosomal miRNA by qRTPCR with 2 extra MM cell lines, H2595 (epithelioid subtype) and HP-1 (biphasic), along with the originally tested Hmeso and H2373, and show (Figure 1B) that miR-16-5p, miR-222-3p, and miR-30a-5p are upregulated in exosomes from both epithelioid subtypes, Hmeso and H2595. The sarcomatoid H2373 showed upregulation only in miR-16-5p and miR-30a-5p, however, no upregulation was observed in the biphasic HP-1. miR-31a-5p was significantly decreased in all MM cancer exosomes.

We also performed qRTPCR to indicate the intracellular levels of each miRNA analyzed to show appropriate comparisons of producer cell quantities versus the amount secreted in exosomes, and found that all were significantly under-expressed as compared to the primary mesothelial cells HPM3 (Figure 1C).

Inhibited exosome secretion of MM cells attenuated tumorigenic properties

Treatment of Hmeso MM cells with small molecule inhibitors GW4869 (GW, 40μM) or combination of Bisindolylmaleimide-I (10μM) with Chloramidine (50μM) (B&C), for 72 hours, resulted in significant reductions in exosome secretion from both treatment groups (Figure 2A & B) as measured by NTA. Subsequent analysis of miR-16-5p provided with confirmation that inhibition of exosome secretion leads to concomitant reduction in

secreted miR-16-5p in addition to increased levels of intracellular miR-16-5p within MM cancer cells (Figure 2C). This result suggests that both inhibitors function differently in their capacity to effect exosome secretion and miR-16 secretion, and that neither inhibitor regulates levels of miR-16. If the inhibitors regulated miR-16 levels directly, we would expect to have seen intracellular levels of the miRNA altering in the same direction as observed in the exosomes; instead, they are increased in the cell and decreased in the exosomes. For further validation, we measured 4 other exosomally secreted miRNAs that were initially identified by microarray, and found that exosome inhibition also leads to reduced levels of exosomal miR-222-3p, miR-30a-5p, miR31-5p, and let-7e-5p (Figure 2D), when measured from direct exosome preparations.

We further measured the viability of MM tumor cells (Hmeso, H2595, and H2373) using the MTS colorimetric assay following 72hr treatment with exosome secretion inhibitors with and without cisplatin. As shown in Figure 3A-C, B&C mediated exosome inhibition caused significant cell death by itself whereas GW did not lead to significant reduction in cell viability (data not shown). Both exosome secretion inhibitors led to significant reductions in cell numbers when combined with a low dose of cisplatin (Figure 3A-C). Furthermore, Annexin-V expression, assayed by flow cytometry, was slightly elevated in all treatment groups compared to control and cisplatin alone, indicating that apoptosis alone does not explain the decrease in cell viability observed upon inhibition of exosome secretion (data not shown).

To elucidate further both the mechanism of reduced number of cells and the effects of miR-16-5p replenishment, we conducted Western blot analysis of miR-16-5p target proteins

CCND1 and BCL2 in exosome secretion inhibited MM cells. Protein levels of both CCND1 and BCL2 decreased in exosome secretion inhibited Hmeso cells (Figure 3D), and CCND1 was decreased in H2595 cells (Figure 3E). BCL2 could not be detected for H2595 cells. At the mRNA level, we showed that exosome inhibition does not affect expression of CCND1 (Figure 3G), although cisplatin does, and that BCL2 levels are significantly reduced upon exosome inhibition and increased with cisplatin (Figure 3G).

The effect of exosome secretion inhibition with and without cisplatin also led to significant reductions in MM cell growth in 3-D spheroid models in both Hmeso (Supplementary Figure 2 A-C) and H2373 cells (Supplementary Figure 2 D-F) as measured by size and MTS assay. Additionally, the migratory and invasive capacity for both Hmeso and H2373 cells was significantly reduced upon inhibition of exosome secretion (Supplementary Figure 3).

Force-feeding MM Cancer Exosomes Back to MM Producer Cells attenuates tumorigenesis

To validate our findings of retention of exosomes and decreased tumor characteristics, we ventured to assess if force-feeding the MM cancer exosomes back to the producer MM cells had a similar effect of reduced tumorigenesis via delivering back their secreted miR-16-5p. As a first step, we validated that MM exosomes interacted with, and were taken up by their own cells by addition of PKH67-labeled exosomes (Figure 4A). All cell lines and their exosomes produced the same results of interaction and staining.

Next, we isolated exosomes from conditioned media after 72 hours in culture for MM exosomes to accumulate. The pelleted exosomes were suspended in 0.5% FBS (exosome-

free) media at three different concentrations: low, medium, and high, based on the amount of media from which the exosomes were pelleted, 5mL, 10mL, and 20mL, respectively. Suspended exosome pellets were added to 3,000 MM cells/well in 96-well plates and incubated overnight. The following day, cells were imaged and assayed for viability by MTS. The MM cells force-fed with their own concentrated exosomes endured significant amounts of cell death as observed by phase-contrast imaging and MTS assay (Figure 4B-G). This was the case for both epithelioid MM subtypes (Hmeso and H2595) and the sarcomatoid subtype (H2373). As an additional control, we added a "mock" group of cells that were exposed to a mixture of exosome-free suspension media and ExoQuick-TC precipitation media after spinning alongside our true exosome isolates to verify that the ExoQuick-TC reagent was not having any effect on the cells. We found that the reagent/media mixture had no significant effect on target cells (Figure 4B, C). Furthermore, we also confirmed that miR-16-5p target protein CCND1 was reduced in abundance. (Figure 4H, I).

To check for the selectivity of exosome force feeding response, we conducted force-feeding of cancer exosomes to non-cancer mesothelial cells (LP9) and also of non-cancer LP9 exosomes to themselves and to the Hmeso MM cancer cells. We found that MM cancer exosomes only killed the MM cancer cells from which they were produced and had no effect on LP9 mesothelial cells. LP9 exosomes had no effect on the proliferation of their producer cells and had a somewhat promotive growth effect on Hmeso cancer cells as analyzed by MTS proliferation assay (Supplementary Figure 4).

miR-16-5p overexpression in MM tumor cells inhibits MM tumorigenesis

To ensure that the above effects seen with exosome secretion inhibition and force-feeding cancer exosomes was in fact due, at least in part, to miR-16-5p levels within the cancer cells we employed direct transfection of miR-16-5p mimics to Hmeso cells. Initial analysis determined that as expected miR-16-5p transfection led to significantly higher levels of intracellular miR-16-5p (Figure 5A). We observed decreased levels of miR-16-5p in exosomes from transfected cells (Figure 5B), suggesting two possibilities; either secretion of exosomes is inhibited in response to transfection or loading of miR-16-5p into exosomes is decreased. Therefore, we conducted NTA from transfected experiments and found that the cells with over-expressed miR-16 upon transfection had no significant change in exosome secretion compared to control cells (Figure 5I).

Further, overexpression of miR-16-5p within the cells resulted in significant reduction in miR-16-5p target proteins CCND1 and BCL2 as analyzed by Western blot (Figure 5C-E). This result also coincided with significantly reduced viable Hmeso cells after miR-16 transfection alone as well as with cisplatin (Figure 5F). To establish that transfection was not specific to only one particular set of miR-16-5p mimics, we transfected cancer cells with two separate miR-16-5p mimics from different lots and observed comparable results. The migratory and invasive capacity of Hmeso cells was also significantly abrogated upon miR-16-5p transfection (Figure 5G, H).

HuR is possibly involved in loading miR-16 into exosomes of MM cells

Based on previous work done by other groups, it has been decisively implicated that the RNA binding protein HuR interacts with miR-16-5p (21). Therefore, a further investigation into exosomal miR-16-5p was to uncover if the RNA binding protein HuR was involved

in loading the miRNA into the vesicles. Immunostaining indicated that HuR was indeed present in the cytoplasm and nucleus of Hmeso MM cells (data not shown). Western blot analysis showed that no measurable amount of HuR was present in MM exosomes (data not shown).

Transfection of siHuR to Hmeso MM cells resulted in significant reduction in HuR protein levels (Figure 6A). Additionally, HuR inhibition caused significantly higher levels of intracellular miR-16-5p and significantly lower levels of exosomal miR-16-5p (Figure 6B,C) suggesting a possible role of HuR in exosomal miRNA chemistry. Interestingly, it has been shown that miR-16 may target the expression of HuR (22), and our data also provides the same evidence by Western blot analysis of Hmeso cells transfected with miR-16-5p (Figure 6D).

Discussion

Malignant mesothelioma is a cancer of mesothelial cells caused by asbestos exposure, with dismal prognosis, and virtually no effective methods of early diagnosis or successful therapeutic approaches. Our hypothesis was to attack these knowledge gaps by embarking to uncover potential miRNA biomarkers and therapeutic targets with the focus on MM tumor exosomes. To date, exosomes have been a trending theme in cancer research and there have been wonderful advancements in understanding cancer through the lens of tumor exosomal miRNAs as possible biomarkers and therapies in many cancers (23-25), however, not much is known about their role(s) in MM.

Our initial findings were surprising in that we observed MM tumor cells secreting significantly higher levels of tumor suppressor miRNAs, particularly miR-16-5p, compared to non-cancer mesothelial cells in exosomes. Previous studies of MM have well-characterized miR-16-5p as both a tumor suppressor by targeting oncogenes BCL2 and CCND1 (26-28), and as a remarkably under-expressed miRNA in MM tumor cells and tissues (10,29). Moreover, miR-16-5p has been reported as a potential therapeutic molecule by restoring its expression in MM (30-32). Based on these previously reported pieces of evidence on the role of miR-16-5p in MM, and our new findings of presence of miR-16-5p in exosomes led us to the innovative hypothesis that MM tumor cells preferentially secrete miR-16-5p, among other tumor suppressors, via exosomes to rid their cancer-killing effects. Similar results have been stated previously in ovarian cancer that may behave similarly in secreting high levels of tumor suppressor miRNAs (33).

The other over-expressed tumor associated exosomal miRNAs also have interesting biological relevance in MM: miR-222-3p is downregulated in MM and is a negative regulator of CDK1 (p27) and is a PTEN suppressor (34); miR-30a-5p (along with miR-222-3p and miR-31-5p), in the same family as miR-30e, is associated with good prognosis when in higher abundance in MM tumors (35); and miR-320 family members are suggested as potential biomarkers for malignant pleural mesothelioma (36).

This set of miRNAs along with miR-16-5p were under-expressed in the MM cells from which they were being secreted. Our findings are supported by literature that MM cells and tumors have low levels of tumor suppressor miRNAs including miR-16-5p (10) as compared to mesothelial cells. This leads us to the possibility that miR-16-5p is not simply

randomly packaged into cancer exosomes due to inherently high abundance in the exosome producer cells, but that it must be systematically sequestered into exosomes by a biologically preferential loading process. The low miR-16-5p in MM tumor cells observed by us and others could be attributed to its increased secretion via exosomes, as opposed to common loss of tumor suppressors via mutation and deletion.

Because of miR-16-5p's tumor suppressive effect and our hypothesis of its preferential secretion from MM tumor cells by exosomes, we aimed to inhibit MM cell tumorigenesis by inhibiting exosome secretion. The idea being that if exosome secretion were significantly down-regulated, that miR-16-5p stores would rebuild in the tumor cells and lead to oncogenic targeting and subsequent cell death.

Based on successful outcomes in the literature we chose to use two separate exosome secretion inhibitory approaches. The neutral sphingolmyelinase-2 inhibitor GW4869, which blocks ceramide production that is needed to bud exosomes inward at the endosomal surface, has been indicated as a useful avenue for blocking exosome secretion as well as increasing the efficacy/reducing chemotherapeutic drug resistance to cisplatin (19,37,38). Further, a combination of two small molecules bisindolylmaleimide-I and chloramidine have also been shown to reduce exosome secretion from cells and increase chemotherapeutic retention (18).

Using GW or B&C treatment of Hmeso cells, led directly to significant reductions in exosomal miR-16-5p along with other miRNA secretions and significantly increased stores of cell cytoplasmic miR-16-5p. The inhibition of exosome release coupled with miR-16-5p retention in cells provided direct evidence that the miRNAs we were investigating were

indeed being released in exosomes, and that we could prevent their preferential release and achieve the goal of miR-16-5p tumor suppressor retention. Also, we see that both inhibitors used, did not appear to negatively regulate the expression of miR-16-5p in the cells, as seen by the increased cellular miR-16-5p, so we can conclude that the drugs work by inhibiting exosomal miR-16-5p secretion and not the levels within the cell. Further confirmation of miR-16-5p retention was assessed by significantly reduced protein abundance of the miR-16-5p targets CCND1 and BCL2 and substantial inhibition in tumorigenesis as measured by loss of cell number, 3D tumor spheroid growth, transformation/colony growth, migratory and invasive capacity of MM tumor cells.

Additionally, we indicate that exosome secretion may play an important role in chemotherapeutic resistance of MM to cisplatin, given that exosome inhibition leads to increased cisplatin-induced cell death in our in vitro studies. In support to our findings, it has been reported before that chemotherapeutic drugs can be lost via exosomes (39,40).

The logical next step for our studies was to show if we can feed back these exosomes to tumor cells and see the similar effects on tumorigenesis and confirm the findings of exosome secretion inhibition. All studies to date on the effect of tumor exosomes has indicated that they are pro-tumorigenic by multiple means (immunosuppression, drug resistance, enhanced tumor growth/proliferation, metastasis, angiogenesis, mesenchymal/fibroblastic transitions etc.) (25,41-47). However, our hypothesis was different because of our intriguing findings that MM tumor exosomes have high volumes of tumor suppressor miRNAs, especially miR-16-5p, and may have a tumor killing effect.

Remarkably, addition of MM exosomes back to the producer MM cells, led to incredible levels of cell death in a dose dependent manner as compared to controls. Further, upon force-feeding of MM cancer exosomes to cancer cells, we see significant decreases in miR-16-5p target oncogenic protein CCND1. This is a vital result in that it shows that we can demonstrate the same result as exosome inhibition but in a separate route. Importantly, MM cancer exosome force-feeding, even more notably, was only seen to kill MM cancer cells and had no effect on normal mesothelial cells. Also, normal mesothelial cell exosomes had no effect on cancer cells or on themselves when force-fed back.

These findings are the first of their kind, to show that not only can a certain cancer's exosomes lead to the death of their producer cells, but they do so in a specific manner that does not affect non-cancer cells. The implications of this are exciting in that they may suggest a very new therapeutic option in MM by targeting tumor cells with their own exosomes. This is especially noteworthy given that the effect of MM exosomes on the cancer cells is implicated in the effect of the tumor suppressor miR-16-5p that is functioning in the exosome inhibition experiments.

We do understand that we are drawing our discussion of force-feeding exosomes based only on one component of what is being redelivered to the tumor cells. In reality, the exosomes will be delivering back a vast array of molecules in combination to the miR-16-5p and other tumor suppressors, and those effects should be considered. Essentially, we know that miR-16-5p plays a role in this complicated biology, but there is likely a lot of interplay with everything else within the MM tumor exosomes that needs to be studied.

Moreover, we recapitulated the same findings from exosome inhibition and force-feeding via direct transfection of miR-16-5p, providing firm evidence that not only is exosome secretion a pro-tumorigenic mechanism of MM by preferential secretion of miR-16-5p but that exosomal miR-16-5p is of potential therapeutic importance as reported before (10,32,48). As a counterintuitive piece of evidence, we did see that although miR-16-5p levels increased intracellularly in MM cancer cells after transfection of miR-16-5p mimic, the levels in their exosomes significantly plummeted. This may suggest that miR-16-5p levels may also play a role in the loading or secretion of exosomes with miR-16-5p within. As NTA analysis showed no significant differences in the number of particles in response to miR-16-5p transfection, there is a further possibility of miR-16-5p exhibiting an effect on packaging/loading system of exosomes.

Here we also intended to unlock the potential mechanism for miR-16 loading into exosomes within MM cancer cells. Based on previous reports of RNA binding protein HuR interaction with miR-16-5p (21) and evident role in exosome secretion of other miRNAs (49), we explored its role in our experimental settings. Significant reduction in HuR expression via siRNA lead to increased amount of miR-16-5p in cells, and significantly reduced the amount secreted in exosomes. This result is evidence that HuR is at least partly involved in the packaging of miR-16-5p into MM cancer exosomes.

Furthermore, previous research has also shown that miR-16 targets the expression of HuR (22), we also provide this same evidence as measured by Western blot analysis, telling us something very interesting about the mechanistics of miR-16-5p exosomal loading and the interplay of HuR. Based on our results thus far, we have drawn together a proposed

mechanism that may be involved where HuR promotes miR-16-5p loading into exosomes, meaning that siHuR knockdown of HuR protein levels leads to reduced exosomal miR-16-5p, and hence increased miR-16-5p in the cell. It is then implicated that miR-16-5p targets HuR expression at the protein level, meaning that high miR-16-5p levels lead to low HuR and therefore, reduced exosomal miR-16-5p, which is exactly what our data represents. Because miR-16-5p upregulation by transfection does not affect exosome secretion by particle number, we know that this effect is limited to the packaging process of exosome cargo. Along this logic, we theorize that MM cancer cells not only have the evolutionary advantage for uncontrolled cell growth because of low intracellular stores of miR-16-5p based on high exosomal removal, but that miR-16-5p itself negatively regulates its own packaging into exosomes by targeting HuR (Supplemental Figure 5).

Taken together, our findings strongly indicate that miR-16-5p is preferentially secreted by MM tumor cells via exosomes in vitro, and by inhibiting exosome secretion, miR-16-5p levels increase thereby reducing oncogenic protein levels and lead to significant loss of tumorigenic capacity of the MM cancer cells. We also indicate that the mechanism of exosomal miR-16-5p secretion is at least somewhat regulated by HuR, and that there is a negative feedback loop involved in this packaging when miR-16-5p levels are increased within the cell.

Altogether, this novel research study provided unprecedented indications that MM tumor exosomes can be used to inhibit tumorigenesis, and this is related to the fact that the MM tumor cells preferentially secrete miR-16-5p through their exosomes to rid themselves of its tumor suppressor function. A recent study published by Guo et al. (50) supports our

concept that autologous tumor cell-derived particles (eg. exosomes) can be a promising therapeutic target for treating malignancies. As discussed above there are limitations to this study as exosomes contain lot more than just tumor suppressor miRNA. Our future endeavors include a series of *in vivo* experiments such as using exosome inhibition and exosome force-feeding in mice allografted with MM tumors to see if any effect can be seen in a whole organism.

Acknowledgments

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Figures and Figure Legends

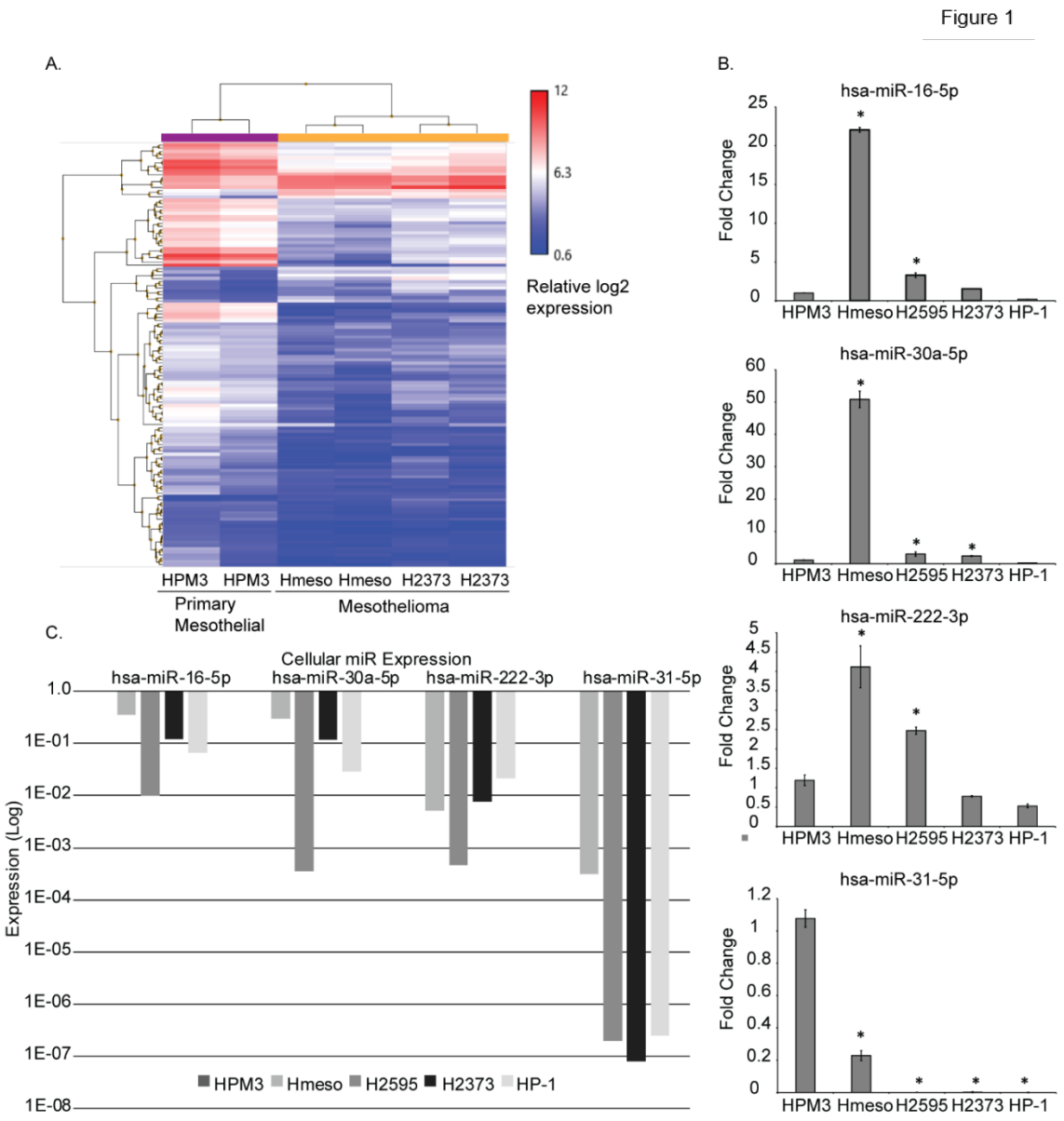


Figure 1. miRNA Array and validation of exosomal miRNAs from mesothelial and mesothelioma cells. A) GeneChip miRNA 4.0 array heat map of miRNA abundances from human mesothelioma cell (Hmeso, H2373) exosomes as compared to normal primary human mesothelial cell (HMP3) exosomes. B) qPCR validation of exosomal miR-16-5p, miR-30a-5p, miR-222-3p, and miR-31-5p in 4 MM cell lines compared to HPM3 cells. C) Cellular miRNA expression of validated exosomal miRNAs in MM cell lines as compared

to HPM3 cells by relative log decrease in expression, with control HPM3 expression levels set to 1.0. Number of replicates are 2 (n=2), data is presented as mean± SEM, and *p ≤ 0.05, by 1-way ANOVA as compared to HPM3 cells.

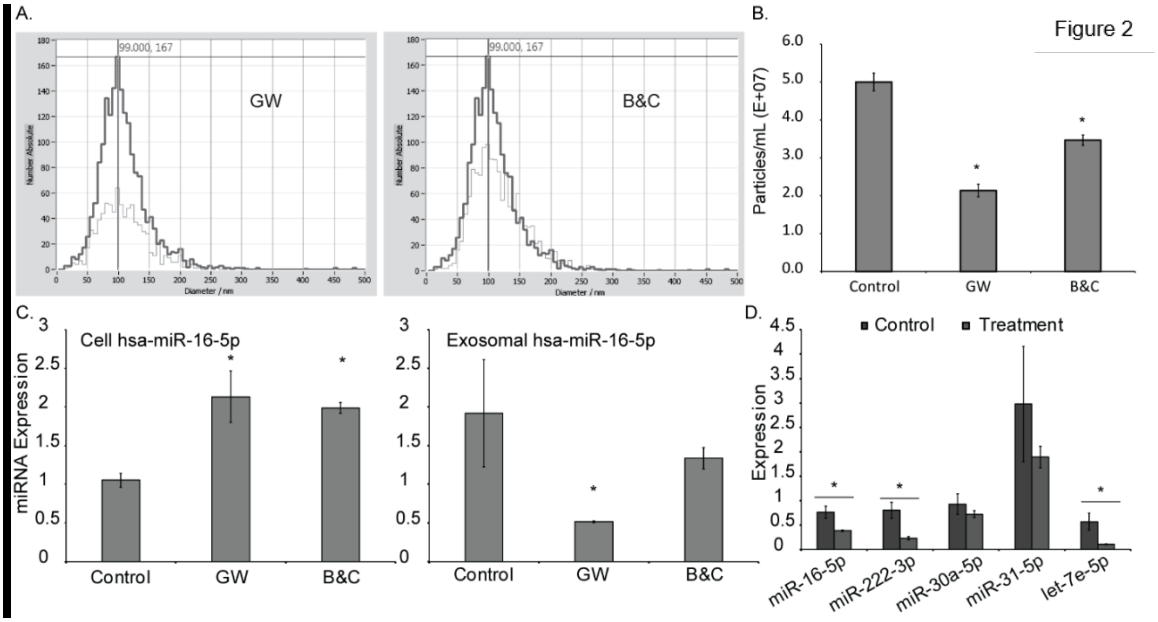


Figure 2. Inhibition of exosome secretion from Hmeso MM cancer cells attenuates exosomal miRNA secretion. A) Nanoparticle tracking analysis plots of control (bold gray) exosomes overlaid with either GW4869 (GW) treated cell exosomes or Bisindolylmaleimide-I with Chloramidine (B&C) treated cell exosomes (light gray). B) Particles/mL of control exosomes compared to both exosome inhibitor treatment groups, n=3/group. C) miR-16 expression in cells and exosomes after exosome secretion inhibition, n=3. D) qPCR validation of other miRNAs being reduced in MM exosomes after exosome inhibition. All miRNA qPCR data is normalized to synthetic spike-in control cel-miR-39-3p, which was added to all exosome or cell isolates prior to RNA isolation. N=2, mean± SEM, and *p ≤ 0.05, by 1-way ANOVA or two-tailed Student's t-test as compared to vehicle treated controls.

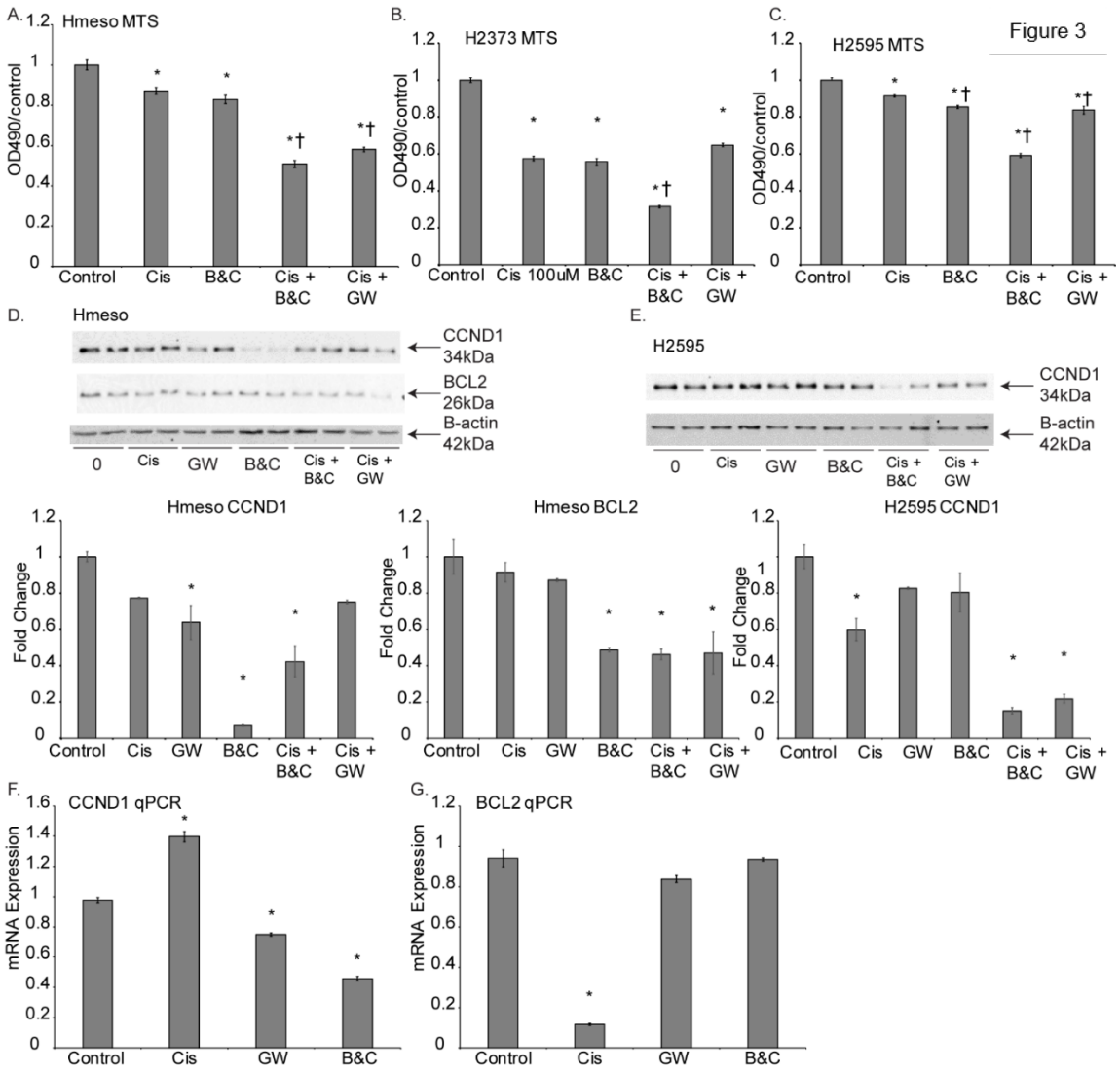


Figure 3. Inhibition of exosome secretion from MM cancer cells reduces proliferation and cellular abundance of oncogenic proteins targeted by miR-16. A) MTS proliferation assay on Hmeso cells after cisplatin treatment, exosome inhibition, or combination of both treatments after 72hr, n=6. B) and C) MTS proliferation assay on H2595 and H2373 cells, respectively, after cisplatin treatment, exosome inhibition, or combination of both treatments after 72hr, n=6 D) Immunoblot of Hmeso cellular proteins and miR-16 targets CCND1 and BCL2, normalized to β-actin. n=2. E) Immunoblot of

H2595 cellular protein and miR-16 target CCND1, normalized to β-actin content. n=2. qPCR of miR-16-5p oncogenes F) CCND1 and G) BCL2 after exosome inhibition, as normalized to HPRT endogenous control. *p ≤ 0.05 as compared to control, † p ≤ 0.05 as compared to cisplatin treated group by 1-way ANOVA.

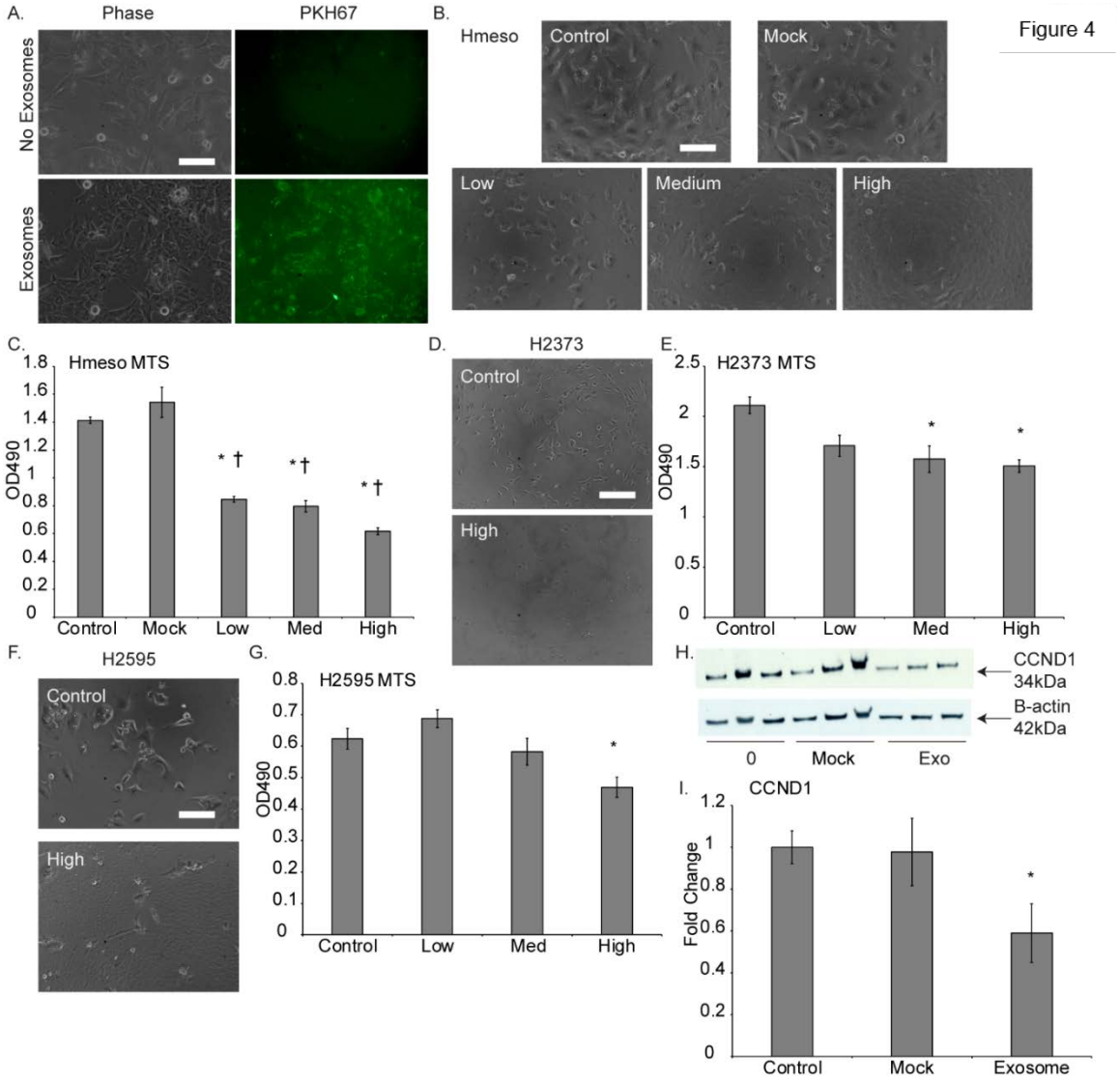


Figure 4. Force-feeding MM cancer cell exosomes back to MM cells leads to cancer cell death. A) PKH67 labeled exosomes from Hmeso cells added to Hmeso cells show uptake/interaction of exosomes with target cancer cells. B) Phase contrast images of Hmeso

cells after addition of three different concentrations of Hmeso exosomes (concentrations based on volume of cell media collected from) or Mock (ExoQuick-TC), and C) MTS proliferation assay of force feeding MM cancer exosomes, n=6. Similar experiments with H2373 cells by D) phase imaging and E) MTS proliferation assay, as well as with H2595 MM cells by F) phase imaging and G) MTS proliferation assay, n=6. Force feeding Hmeso exosomes to Hmeso MM cells H) significantly reduced protein levels of CCND1 as analyzed by Western blot analysis. Western blot images shown are representative images and quantitation graphs of I) CCND1 are combined quantitation of 3 repeated experiments. N=3, Phase contrast images were taken with 40× objective lens, scale bar = 100μm, mean $\pm$ SEM, *$p \leq 0.05$ as compared to control and † $p \leq 0.05$ as compared to Mock by 1-way ANOVA.

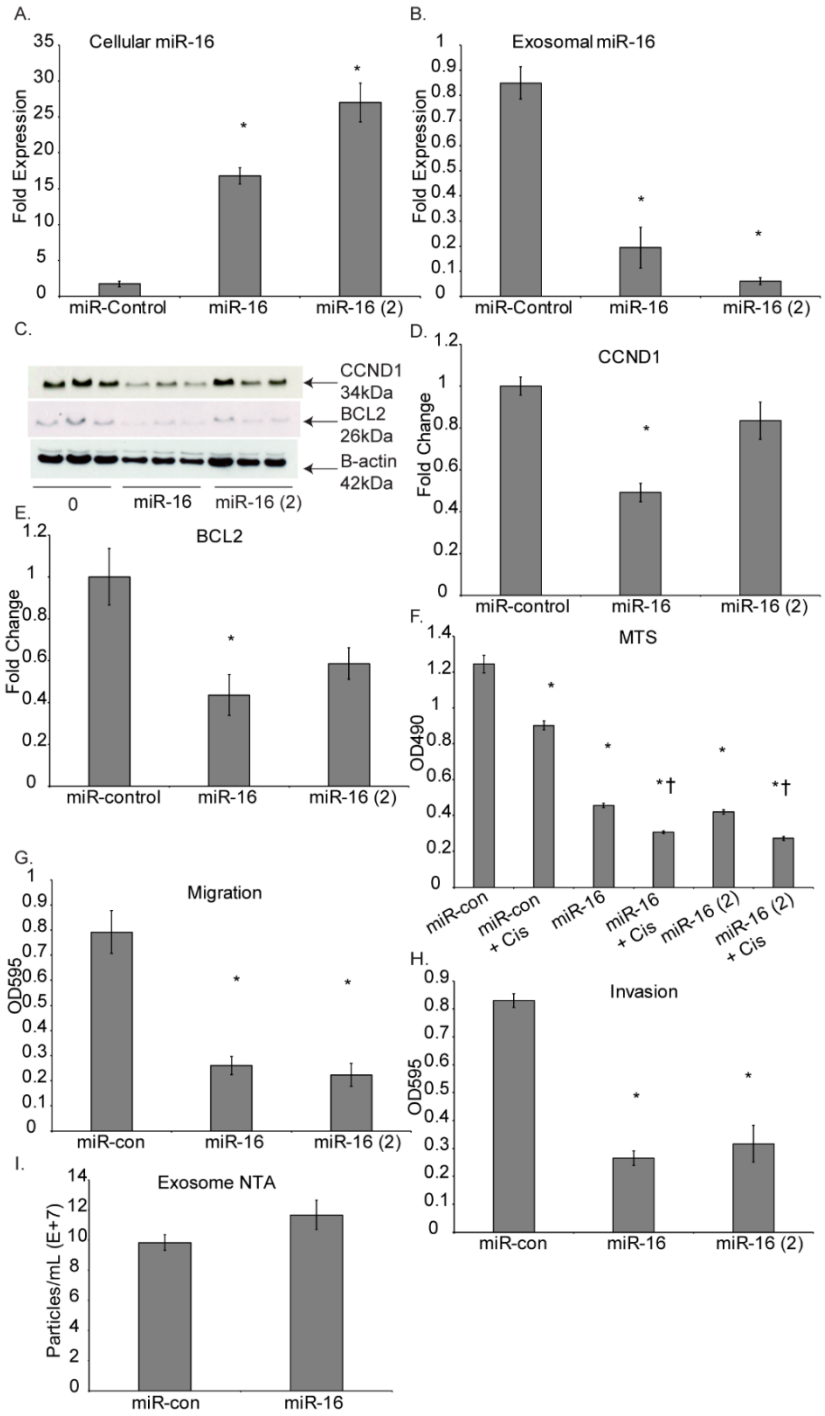


Figure 5. miR-16 overexpression in Hmeso cells leads to decreased MM cancer cell proliferation and protein abundance of CCND1 and BCL2. A) Cellular levels of miR-16 increased after transfection, whereas, contradictorily, B) miR-16 levels in exosomes

significantly decreased upon transfection of miR-16 into Hmeso cells. n=3. The target proteins of miR-16 C) (immunoblot), D) CCND1 and E) BCL2 were reduced after miR-16 transfection. Transfection of miR-16 also resulted in F) significantly reduced proliferation of MM cells by MTS, and increased cell death by cisplatin (n=6), along with attenuated capabilities of G) migration (n=3) and H) invasion (n=3). I) miR-16 transfection had no effect on secreted exosome numbers as assessed by NTA. All miRNA qPCR data is normalized to synthetic spike-in control cel-miR-39-3p, which was added to all exosome or cell isolates prior to RNA isolation. Mean $\pm$ SEM, *$p \leq 0.05$ as compared to control and †$p \leq 0.05$ as compared to cisplatin by 1-way ANOVA.

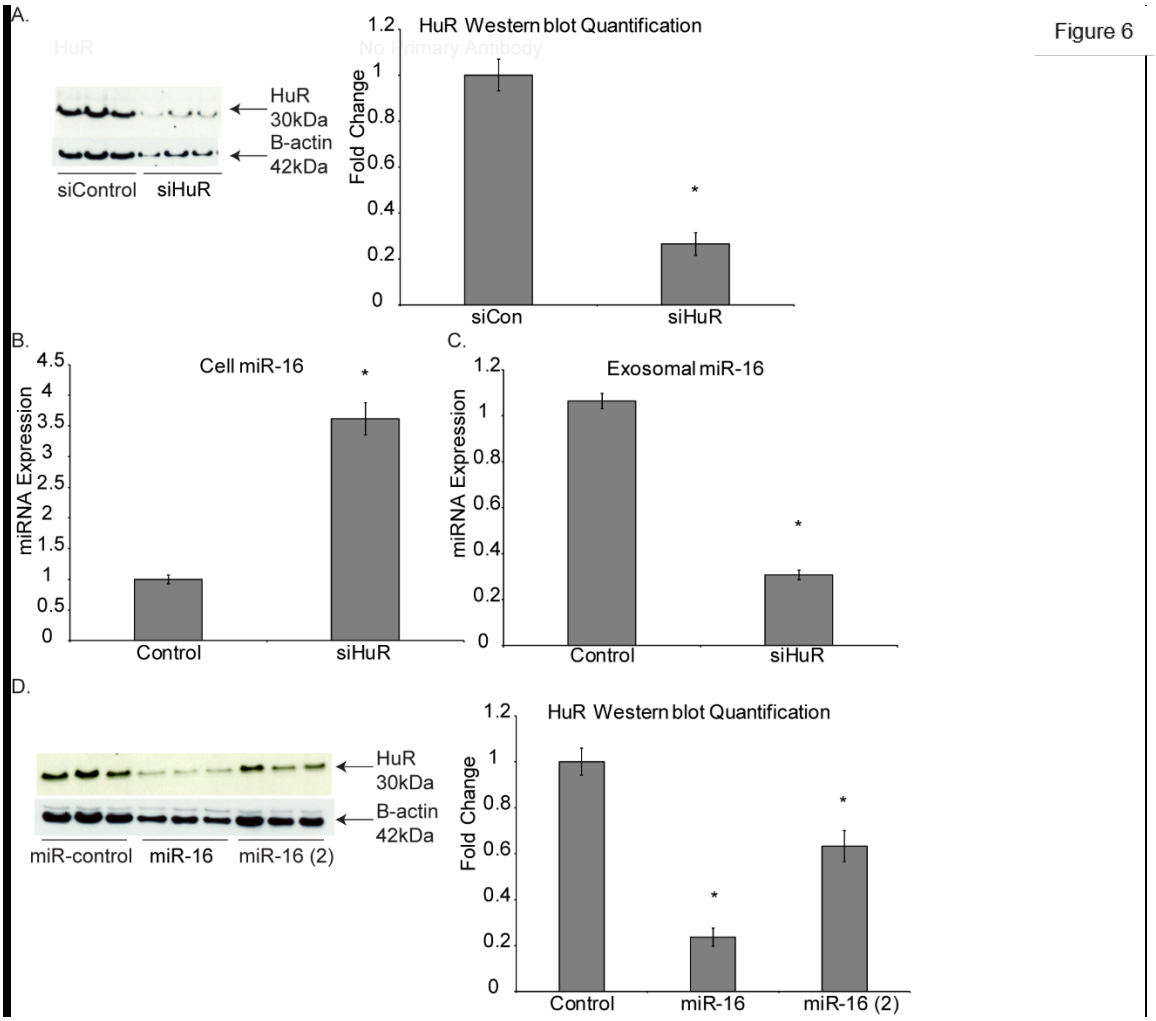
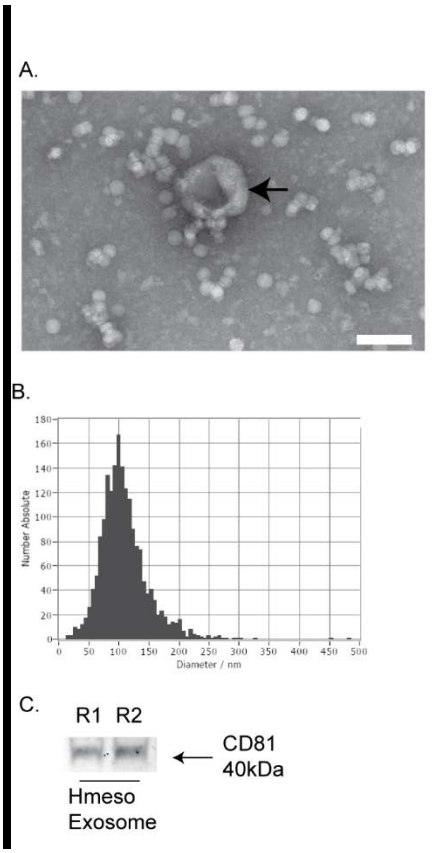
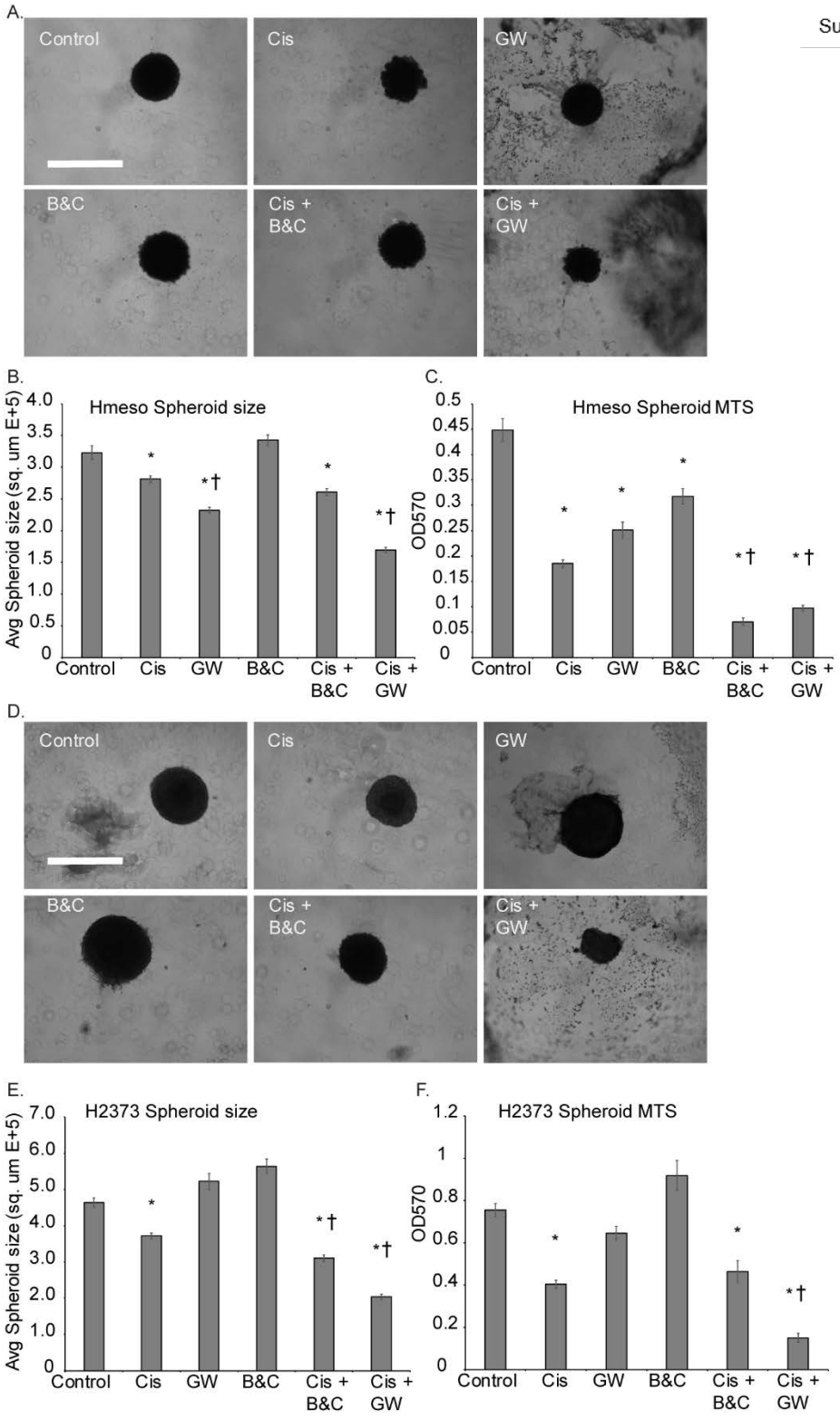


Figure 6. HuR is involved in the exosomal secretion of miR-16 from MM cells. A) Transfection of siHuR significantly reduced the protein abundance of HuR as analyzed by Western blot analysis B), and led to significantly increased cellular expression of miR-16 and C) significantly decreased expression of miR-16 in exosomes. D) Transfection of miR-16 mimic similarly induced significant reductions in HuR protein levels as analyzed by Western blot analysis (using the same immunoblot as Figure 5, therefore B-actin is the same). n=3. mean $\pm$ SEM, *$p \leq 0.05$ as compared to control by 1-way ANOVA

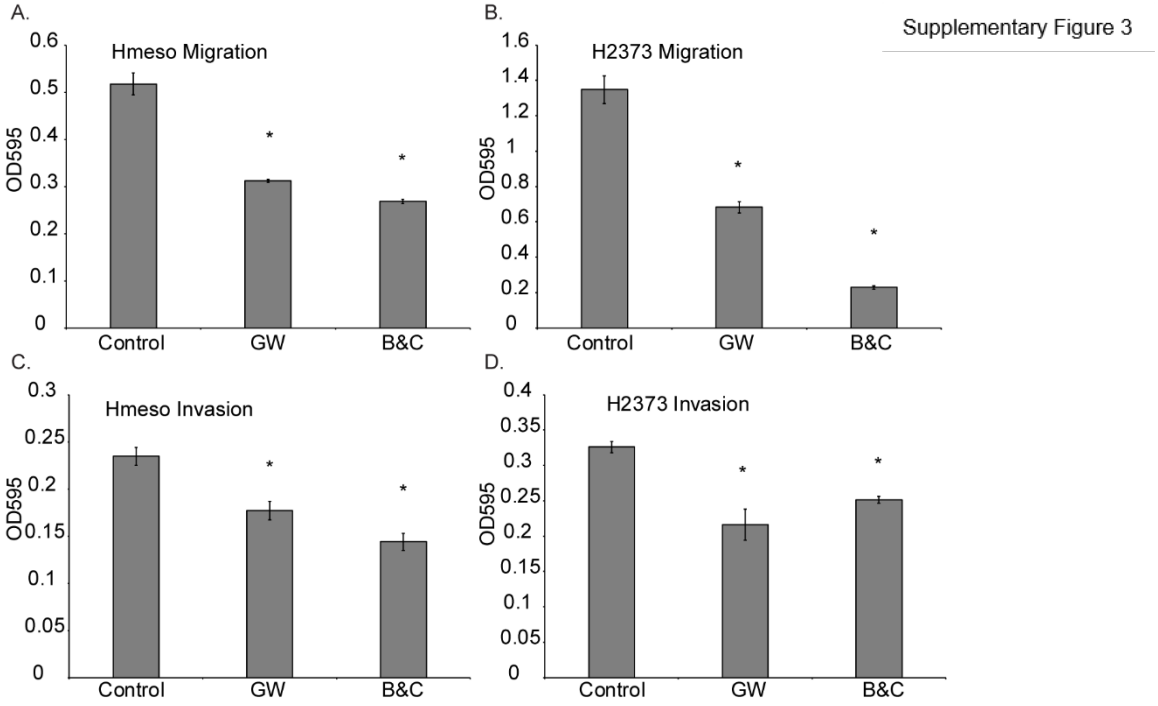


Supplementary Figure 1. Hmeso cell exosome characterization. A) TEM showing exosome membrane-bound structures and size range; indicated by arrow in middle of field. Scale bars, 100 nm. B) Nanoparticle Tracking Analysis (NTA) indicating exosome size distribution and concentration of particles. C) Western blot analysis for presence of exosome marker CD81 from two replicate (R1 and R2) exosome preparations from Hmeso conditioned cell culture supernatant, normalized by equal volume of exosomal input from equal cell number.



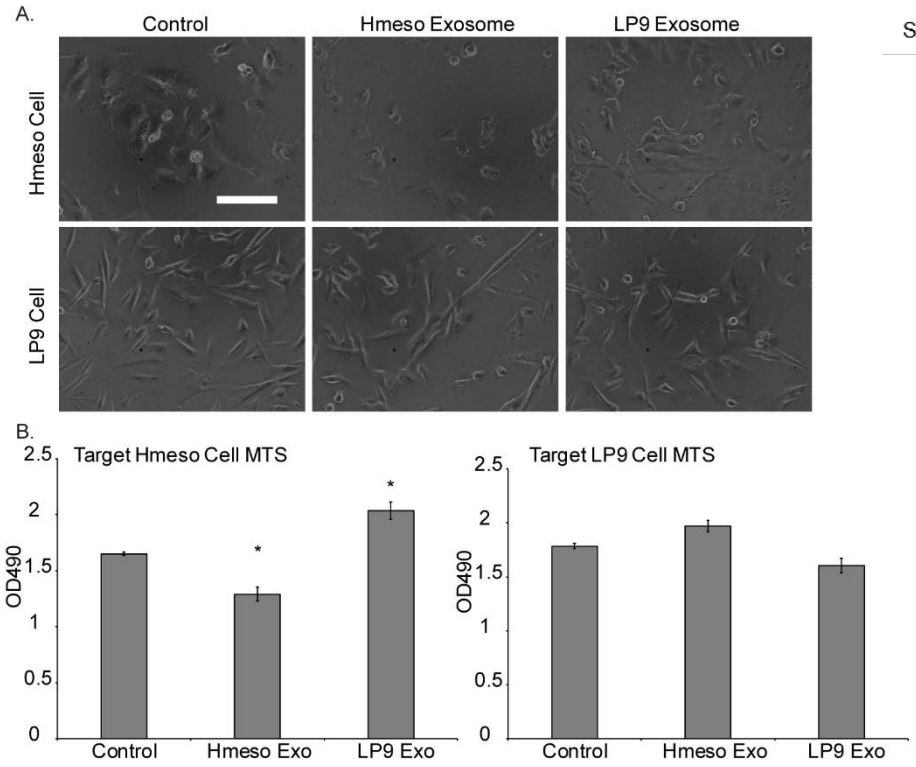
Supplementary Figure 2. Exosome secretion inhibition from MM cancer cells inhibits

tumor 3D spheroid growth. A) Hmeso cells were grown as 3D spheroids as described in materials and methods section and treated with cisplatin (Cis, 20µM), GW4869 (GW, 40µM), combination of Bisindolylmaleimide-I 10µM with Chloramidine 50µM (B&C), or combinations. Phase contrast images were taken after 6 days of growth with 20× objective lens, scale bar, 500µM B) 3D spheroid sizes were measured in ImageJ, and C) solubilized and measured for proliferation using MTS assay. D-F) Similar experiments with H2373 cells n=3, mean± SEM, *p ≤ 0.05 as compared to vehicle control and † p ≤ 0.05 as compared to Cis alone by 1-way ANOVA



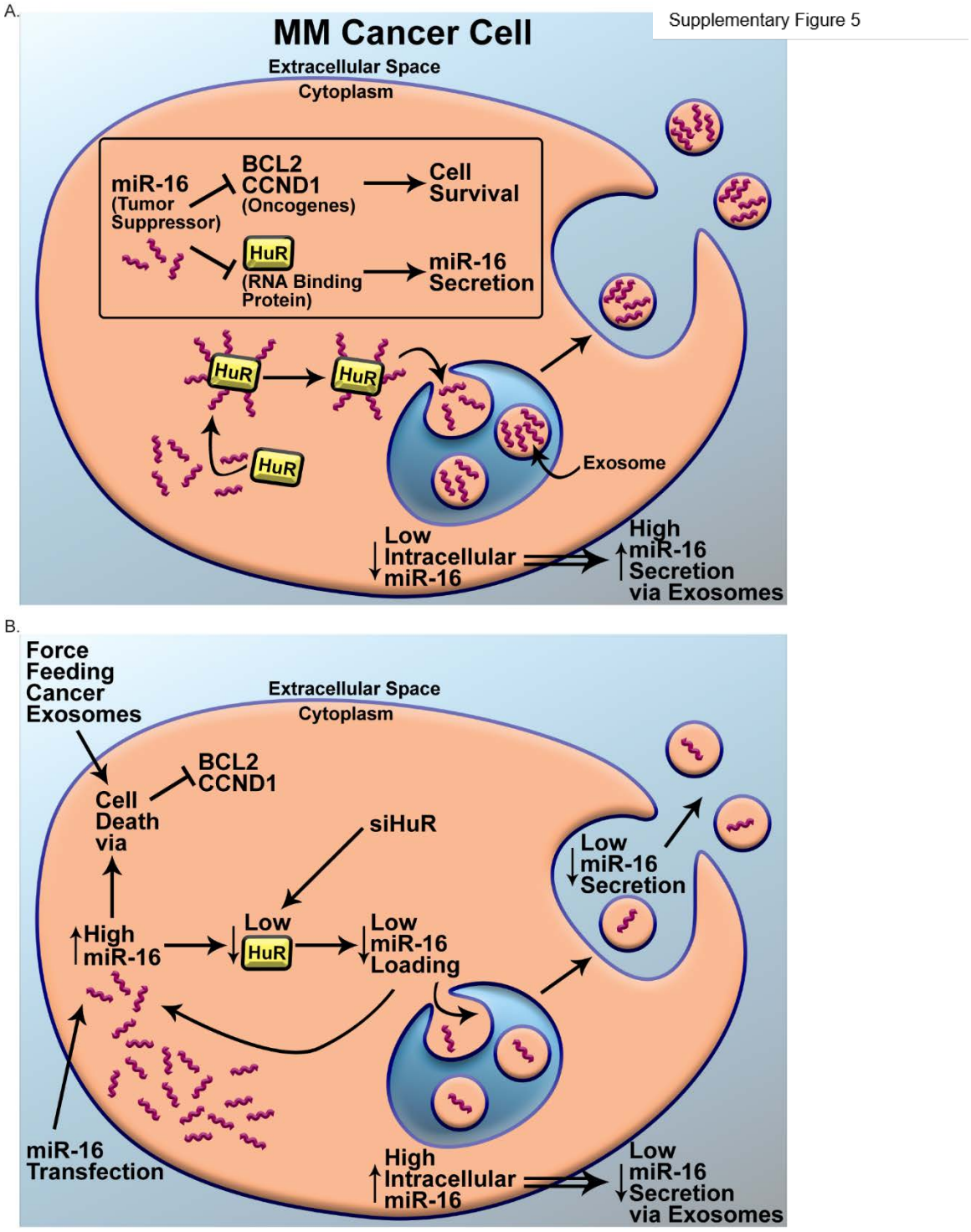
Supplementary Figure 3. Exosome secretion inhibition from MM cancer cells attenuated migration and invasion. MM cells were treated with exosome secretion inhibitors and then put on tranwells to assess migration /invasion after 72 h as described in

the materials and method section. Inhibition of exosome secretion attenuated migration (A, B) and invasion (C, D) of Hmeso and H2373 MM cells. n=3, mean± SEM, *p ≤ 0.05 as compared to vehicle control by 1-way ANOVA.



Supplementary Figure 4

Supplementary Figure 4. Exosome force-feeding leads to cancer cell specific effects. A) Phase contrast images of control and force-fed mesothelial (LP9) or mesothelioma (Hmeso) cells in various combinations and B) MTS proliferation assay of force-feeding MM cancer exosomes and LP9 mesothelial cell exosomes to either target Hmeso MM cells or target LP9 cells. n=6, Phase contrast images were taken with 40× objective lens, scale bar = 100µm, mean± SEM, *p ≤ 0.05 as compared to control by 1-way ANOVA.



Supplementary Figure 5

Supplementary Figure 5. Schematic representation of hypothesized mechanism of exosomal miR-16-5p secretion in MM cancer cells. According to our results, along with

published literature, we concluded that in malignant mesothelioma (MM) cancer cells A) miR-16-5p is loaded into exosomes, with the help of the RNA binding protein HuR, and is secreted at high levels in those exosomes. Further, we know that miR-16-5p, a potent tumor suppressor, acts to block gene expression of oncogenic BCL2 and CCND1, and that miR-16-5p negatively regulates HuR, leading to the circumstance that MM cancer cells have low intracellular miR-16-5p and high exosomal miR-16-5p. Our further experiments indicated that: B) force-feeding of MM cancer exosomes to MM cancer cells leads to cell death by blocking oncogenic protein abundances of BCL2 and CCND1; miR-16-5p transfection leads to low HuR levels therefore low miR-16-5p secretion and replenished miR-16-5p intracellular stores, also leading to cell death by BCL2 and CCND1 regulation; and that direct regulation of HuR by siHuR can recapitulate this same cycle of potential therapeutic targeting to increase miR-16-5p expression in cancer cells. Exosome inhibition was omitted from the schematic for clarity and because the role of inhibiting exosome secretion in MM cancer cells on HuR levels has not been elucidated.

Supplementary Table 1. List of differentially expressed miRNAs in MM cancer exosomes compared to non-cancer mesothelial cell exosomes.

Transcript ID (Array Design)	Mesothelioma Avg (log2)	Normal Avg (log2)	Fold Change	P-val
hsa-miR-30a-5p	6.12	2.79	10.05	4.58E-06
hsa-miR-16-5p	6.89	4.18	6.52	0.0015
hsa-miR-92b-3p	4.78	2.4	5.2	0.0257
hsa-miR-1268a	5.76	3.66	4.31	0.0433
hsa-miR-25-3p	4.57	2.52	4.14	0.0361
hsa-miR-320e	5.54	3.57	3.92	4.30E-06
hsa-miR-222-3p	7.37	5.49	3.7	0.0036
hsa-miR-15b-5p	4.26	2.41	3.62	0.0063
hsa-miR-92a-3p	9.96	8.13	3.56	0.0185
hsa-miR-320d	7.71	5.96	3.36	0.0009
hsa-miR-320c	9.46	7.8	3.17	0.0095
hsa-miR-20a-5p	5	3.47	2.88	0.0191
hsa-miR-320a	9.5	8.02	2.79	0.0035
hsa-miR-320b	9.45	8	2.72	0.0049
hsa-miR-4445-3p	4.06	2.65	2.66	0.0231
hsa-miR-7114-5p	2.77	1.39	2.6	0.0246

hsa-miR-548ap-3p	3.36	2.17	2.28	0.0206
hsa-mir-7515	4.36	3.31	2.08	0.0447
hsa-miR-744-5p	3.55	2.54	2.02	0.0365
hsa-miR-3910	2.2	1.2	2.01	0.0018
hsa-miR-885-3p	2.63	3.67	-2.05	0.0165
hsa-miR-4655-5p	1.93	2.97	-2.05	0.0203
hsa-miR-6124	1.35	2.4	-2.07	0.0123
hsa-miR-4681	1.67	2.73	-2.09	0.0029
hsa-miR-6820-5p	2.35	3.45	-2.14	0.0178
hsa-miR-5196-5p	1.48	2.6	-2.17	0.004
hsa-miR-6875-5p	1.15	2.27	-2.17	0.0065
hsa-miR-3162-5p	1.47	2.6	-2.18	0.0011
hsa-miR-3619-5p	1.97	3.1	-2.19	0.0314
hsa-miR-6775-5p	4.05	5.2	-2.21	0.0185
hsa-miR-6127	1.69	2.84	-2.23	0.0054
hsa-miR-4707-5p	3.93	5.12	-2.29	0.0027
hsa-miR-6125	5.39	6.61	-2.32	0.0341
hsa-miR-1233-5p	3.35	4.57	-2.32	0.0156
hsa-miR-6729-5p	6.31	7.53	-2.33	0.0026
hsa-miR-1207-5p	3.64	4.86	-2.33	0.0352
hsa-miR-498	3.25	4.48	-2.33	0.0298

hsa-miR-6077	1.69	2.94	-2.38	0.0015
hsa-mir-4281	2.36	3.62	-2.39	8.84E-05
hsa-miR-4787-5p	6.9	8.18	-2.44	0.0014
hsa-miR-135a-3p	1.06	2.35	-2.46	0.0017
hsa-miR-7109-5p	1.56	2.86	-2.47	0.0038
hsa-miR-5787	7.89	9.23	-2.54	0.0054
hsa-miR-210-3p	3.87	5.21	-2.54	0.042
hsa-miR-6724-5p	3.95	5.37	-2.66	0.0123
hsa-mir-6800	3.31	4.73	-2.68	5.50E-05
hsa-miR-6848-5p	2.59	4.02	-2.69	0.0304
hsa-miR-6787-5p	2.95	4.38	-2.7	0.0116
hsa-miR-6782-5p	3.33	4.79	-2.75	0.0049
hsa-miR-4665-5p	1.5	2.95	-2.75	0.006
hsa-miR-6850-5p	3.37	4.84	-2.76	0.0012
hsa-miR-5100	1.56	3.08	-2.87	0.0156
hsa-miR-6791-5p	3.72	5.25	-2.89	0.0026
hsa-miR-204-3p	1.59	3.16	-2.97	0.0181
hsa-miR-4749-5p	2.69	4.33	-3.13	0.0001
hsa-miR-4417	0.83	2.48	-3.14	0.0002
hsa-miR-4758-5p	2.39	4.04	-3.14	0.0138
hsa-miR-6727-5p	5.4	7.11	-3.28	0.0014
hsa-miR-6858-5p	3.49	5.21	-3.28	0.0005

hsa-mir-6800	3.25	4.98	-3.33	4.85E-05
hsa-miR-1227-5p	3.16	4.92	-3.4	0.0026
hsa-miR-4674	1.86	3.63	-3.41	0.0051
hsa-miR-6824-5p	1.57	3.37	-3.48	0.008
hsa-miR-8069	4.97	6.77	-3.49	0.0251
hsa-miR-7108-5p	3.58	5.39	-3.5	0.0116
hsa-miR-1915-3p	4.95	6.76	-3.51	0.0014
hsa-miR-4689	1.3	3.13	-3.55	0.0325
hsa-miR-149-3p	4.62	6.46	-3.59	0.0056
hsa-miR-6786-5p	5.73	7.6	-3.64	0.0018
hsa-miR-1909-3p	1.2	3.07	-3.66	0.0048
hsa-let-7e-5p	2.04	3.96	-3.79	5.73E-05
hsa-miR-4492	2.44	4.37	-3.82	0.0005
hsa-mir-4466	1.92	3.89	-3.93	0.0022
hsa-miR-6891-5p	1.87	3.85	-3.94	0.0126
hsa-miR-3665	6	8	-4	0.0099
hsa-miR-3196	4.67	6.68	-4.01	0.0128
hsa-miR-6743-5p	3.05	5.06	-4.02	0.023
hsa-miR-3960	7.34	9.36	-4.06	0.0025
hsa-miR-6716-5p	1.38	3.41	-4.07	0.0093
hsa-miR-3180-3p	1.91	3.95	-4.1	0.0182
hsa-miR-6805-5p	3.42	5.46	-4.13	0.0285

hsa-miR-6771-5p	1.86	3.91	-4.13	0.0016
hsa-miR-6722-3p	2.14	4.25	-4.33	0.0021
hsa-miR-7110-5p	2.2	4.32	-4.37	0.002
hsa-miR-8072	5.39	7.6	-4.61	0.0036
hsa-miR-3656	4.76	7.04	-4.84	0.0013
hsa-miR-6802-5p	1.21	3.49	-4.84	0.0171
hsa-miR-4651	3.41	5.7	-4.9	0.0007
hsa-miR-6798-5p	3.16	5.51	-5.1	0.0052
hsa-miR-1273g-3p	3.74	6.11	-5.19	0.0105
hsa-miR-4649-5p	2.3	4.68	-5.19	0.001
hsa-miR-6803-5p	5.16	7.57	-5.31	3.29E-05
hsa-miR-1225-5p	1.6	4.01	-5.32	0.0334
hsa-miR-4488	4.71	7.19	-5.54	0.0111
hsa-miR-6869-5p	4.81	7.28	-5.56	0.0025
hsa-miR-3648	1.55	4.08	-5.76	4.26E-05
hsa-miR-3663-3p	1.46	4.04	-5.99	1.64E-05
hsa-miR-4466	5.7	8.33	-6.18	0.0003
hsa-miR-6752-5p	3.61	6.24	-6.19	0.0015
hsa-miR-6088	4.96	7.6	-6.23	0.0001
hsa-miR-4433b-3p	2.4	5.09	-6.44	0.0049

hsa-miR-4508	4.51	7.22	-6.56	6.58E-05
hsa-miR-6087	6.18	8.9	-6.58	5.78E-05
hsa-miR-1228-5p	3.89	6.67	-6.84	0.0003
hsa-miR-6789-5p	1.99	4.77	-6.87	0.0005
hsa-miR-3940-5p	3.09	5.9	-7.03	0.0088
hsa-miR-6749-5p	1.84	4.71	-7.3	0.0036
hsa-miR-4463	2.47	5.4	-7.61	0.0009
hsa-miR-4687-3p	3.02	6.04	-8.13	0.002
hsa-miR-4497	7.03	10.11	-8.45	0.0012
hsa-miR-6765-5p	2.96	6.1	-8.82	0.0003
hsa-miR-6090	6.32	9.5	-9.06	0.0003
hsa-miR-6089	6.5	9.77	-9.63	0.0002
hsa-mir-6089-1	2.4	5.73	-10.1	0.0002
hsa-mir-6089-2	2.4	5.73	-10.1	0.0002
hsa-miR-1343-5p	1.72	5.07	-10.14	3.07E-06
hsa-miR-31-5p	1.32	4.83	-11.4	0.0002
hsa-miR-328-5p	2.98	6.54	-11.81	0.0003
hsa-miR-3197	3.95	7.8	-14.5	0.0285
hsa-miR-1237-5p	4.63	8.53	-14.86	1.97E-05
hsa-miR-762	4.39	8.37	-15.73	4.18E-05
hsa-miR-4745-5p	2.59	6.61	-16.25	0.001
hsa-miR-4516	3.26	7.34	-16.88	0.0003

hsa-miR-6816-5p	3.01	7.58	-23.72	0.0001
hsa-miR-6780b-5p	2.38	7.17	-27.84	2.72E-06
hsa-miR-3178	4.72	9.54	-28.29	0.0012
hsa-miR-4281	1.8	6.94	-35.37	4.75E-06
hsa-miR-4532	1.83	7.14	-39.78	9.00E-07
hsa-miR-6126	4.73	10.36	-49.59	8.07E-05
hsa-miR-4484	3.06	10.06	-128.2	3.05E-06

References

1. Mossman BT, Shukla A, Heintz NH, Verschraegen CF, Thomas A, Hassan R. New insights into understanding the mechanisms, pathogenesis, and management of malignant mesotheliomas. The American journal of pathology **2013**;182(4):1065-77 doi 10.1016/j.ajpath.2012.12.028.
2. Rossini M, Rizzo P, Bononi I, Clementz A, Ferrari R, Martini F*, et al.* New Perspectives on Diagnosis and Therapy of Malignant Pleural Mesothelioma. Frontiers in oncology **2018**;8:91 doi 10.3389/fonc.2018.00091.
3. Yap TA, Aerts JG, Popat S, Fennell DA. Novel insights into mesothelioma biology and implications for therapy. Nature reviews Cancer **2017**;17(8):475-88 doi 10.1038/nrc.2017.42.
4. Munson P, Shukla A. Exosomes: Potential in Cancer Diagnosis and Therapy. Medicines **2015**;2(4):310-27 doi 10.3390/medicines2040310.
5. Yu DD, Wu Y, Shen HY, Lv MM, Chen WX, Zhang XH*, et al.* Exosomes in Development, Metastasis and Drug Resistance of Breast Cancer. Cancer science **2015** doi 10.1111/cas.12715.
6. Brinton LT, Sloane HS, Kester M, Kelly KA. Formation and role of exosomes in cancer. Cellular and molecular life sciences : CMLS **2015**;72(4):659-71 doi 10.1007/s00018-014-1764-3.
7. Bard MP, Hegmans JP, Hemmes A, Luider TM, Willemsen R, Severijnen LA*, et al.* Proteomic analysis of exosomes isolated from human malignant pleural effusions. American journal of respiratory cell and molecular biology **2004**;31(1):114-21 doi 10.1165/rcmb.2003-0238OC.
8. Hegmans JP, Bard MP, Hemmes A, Luider TM, Kleijmeer MJ, Prins JB*, et al.* Proteomic analysis of exosomes secreted by human mesothelioma cells. The American journal of pathology **2004**;164(5):1807-15 doi 10.1016/s0002-9440(10)63739-x.
9. Mahaweni NM, Kaijen-Lambers ME, Dekkers J, Aerts JG, Hegmans JP. Tumour-derived exosomes as antigen delivery carriers in dendritic cell-based immunotherapy for malignant mesothelioma. Journal of extracellular vesicles **2013**;2 doi 10.3402/jev.v2i0.22492.
10. Reid G. MicroRNAs in mesothelioma: from tumour suppressors and biomarkers to therapeutic targets. Journal of thoracic disease **2015**;7(6):1031-40 doi 10.3978/j.issn.2072-1439.2015.04.56.
11. Cavalleri T, Angelici L, Favero C, Dioni L, Mensi C, Bareggi C*, et al.* Plasmatic extracellular vesicle microRNAs in malignant pleural mesothelioma and asbestos-exposed subjects suggest a 2-miRNA signature as potential biomarker of disease. PLoS ONE **2017**;12(5):e0176680 doi 10.1371/journal.pone.0176680.
12. Munson P, Lam YW, MacPherson M, Beuschel S, Shukla A. Mouse serum exosomal proteomic signature in response to asbestos exposure. Journal of cellular biochemistry **2018** doi 10.1002/jcb.26863.
13. Munson P, Lam YW, Dragon J, MacPherson M, Shukla A. Exosomes from asbestos-exposed cells modulate gene expression in mesothelial cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology **2018**:fj201701291RR doi 10.1096/fj.201701291RR.

14. Pass HI, Stevens EJ, Oie H, Tsokos MG, Abati AD, Fetsch PA*, et al.* Characteristics of nine newly derived mesothelioma cell lines. The Annals of thoracic surgery **1995**;59(4):835-44.
15. Reale FR, Griffin TW, Compton JM, Graham S, Townes PL, Bogden A. Characterization of a human malignant mesothelioma cell line (H-MESO-1): a biphasic solid and ascitic tumor model. Cancer research **1987**;47(12):3199-205.
16. Shukla A, Miller JM, Cason C, Sayan M, MacPherson MB, Beuschel SL*, et al.* Extracellular signal-regulated kinase 5: a potential therapeutic target for malignant mesotheliomas. Clinical cancer research : an official journal of the American Association for Cancer Research **2013**;19(8):2071-83 doi 10.1158/1078-0432.CCR-12-3202.
17. Hillegass JM, Blumen SR, Cheng K, MacPherson MB, Alexeeva V, Lathrop SA*, et al.* Increased efficacy of doxorubicin delivered in multifunctional microparticles for mesothelioma therapy. International journal of cancer **2011**;129(1):233-44 doi 10.1002/ijc.25666.
18. Kosgodage US, Trindade RP, Thompson PR, Inal JM, Lange S. Chloramidine/Bisindolylmaleimide-I-Mediated Inhibition of Exosome and Microvesicle Release and Enhanced Efficacy of Cancer Chemotherapy. International journal of molecular sciences **2017**;18(5) doi 10.3390/ijms18051007.
19. Essandoh K, Yang L, Wang X, Huang W, Qin D, Hao J*, et al.* Blockade of exosome generation with GW4869 dampens the sepsis-induced inflammation and cardiac dysfunction. Biochimica et biophysica acta **2015**;1852(11):2362-71 doi 10.1016/j.bbadis.2015.08.010.
20. Thompson JK, Shukla A, Leggett AL, Munson PB, Miller JM, MacPherson MB*, et al.* Extracellular signal regulated kinase 5 and inflammasome in progression of mesothelioma. Oncotarget **2018**;9(1):293-305 doi 10.18632/oncotarget.22968.
21. Young LE, Moore AE, Sokol L, Meisner-Kober N, Dixon DA. The mRNA stability factor HuR inhibits microRNA-16 targeting of COX-2. Molecular cancer research : MCR **2012**;10(1):167-80 doi 10.1158/1541-7786.mcr-11-0337.
22. Xu F, Zhang X, Lei Y, Liu X, Liu Z, Tong T*, et al.* Loss of Repression of HuR Translation by miR-16 May Be Responsible for the Elevation of HuR in Human Breast Carcinoma. Journal of cellular biochemistry **2010**;111(3):727-34 doi doi:10.1002/jcb.22762.
23. Rabinowits G, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. Clinical lung cancer **2009**;10(1):42-6 doi 10.3816/CLC.2009.n.006.
24. Chevillet JR, Kang Q, Ruf IK, Briggs HA, Vojtech LN, Hughes SM*, et al.* Quantitative and stoichiometric analysis of the microRNA content of exosomes. Proceedings of the National Academy of Sciences of the United States of America **2014**;111(41):14888-93 doi 10.1073/pnas.1408301111.
25. Melo SA, Sugimoto H, O'Connell JT, Kato N, Villanueva A, Vidal A*, et al.* Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. Cancer cell **2014**;26(5):707-21 doi 10.1016/j.ccell.2014.09.005.
26. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M*, et al.* miR-15 and miR-16 induce apoptosis by targeting BCL2. Proceedings of the National Academy of Sciences of the United States of America **2005**;102(39):13944-9 doi 10.1073/pnas.0506654102.

27. Wang DW, Wang YQ, Shu HS. MiR-16 inhibits pituitary adenoma cell proliferation via the suppression of ERK/MAPK signal pathway. European review for medical and pharmacological sciences **2018**;22(5):1241-8 doi 10.26355/eurrev_201803_14464.
28. Kao SC, Cheng YY, Williams M, Kirschner MB, Madore J, Lum T*, et al.* Tumor Suppressor microRNAs Contribute to the Regulation of PD-L1 Expression in Malignant Pleural Mesothelioma. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer **2017**;12(9):1421-33 doi 10.1016/j.jtho.2017.05.024.
29. De Santi C, Melaiu O, Bonotti A, Cascione L, Di Leva G, Foddis R*, et al.* Deregulation of miRNAs in malignant pleural mesothelioma is associated with prognosis and suggests an alteration of cell metabolism. Sci Rep **2017**;7(1):3140 doi 10.1038/s41598-017-02694-0.
30. Reid G, Pel ME, Kirschner MB, Cheng YY, Mugridge N, Weiss J*, et al.* Restoring expression of miR-16: a novel approach to therapy for malignant pleural mesothelioma. Annals of oncology : official journal of the European Society for Medical Oncology **2013**;24(12):3128-35 doi 10.1093/annonc/mdt412.
31. Fennell D. miR-16: expanding the range of molecular targets in mesothelioma. The Lancet Oncology **2017** doi 10.1016/s1470-2045(17)30596-x.
32. Viteri S, Rosell R. An innovative mesothelioma treatment based on miR-16 mimic loaded EGFR targeted minicells (TargomiRs). Translational Lung Cancer Research **2017**:S1-S4.
33. Kanlikilicer P, Rashed MH, Bayraktar R, Mitra R, Ivan C, Aslan B*, et al.* Ubiquitous Release of Exosomal Tumor Suppressor miR-6126 from Ovarian Cancer Cells. Cancer research **2016**;76(24):7194-207 doi 10.1158/0008-5472.can-16-0714.
34. Truini A, Coco S, Alama A, Genova C, Sini C, Dal Bello MG*, et al.* Role of microRNAs in malignant mesothelioma. Cellular and Molecular Life Sciences **2014**;71(15):2865-78 doi 10.1007/s00018-014-1584-5.
35. Birnie KA, Prêle CM, Thompson PJ, Badrian B, Mutsaers SE. Targeting microRNA to improve diagnostic and therapeutic approaches for malignant mesothelioma. Oncotarget **2017**;8(44):78193-207 doi 10.18632/oncotarget.20409.
36. Quinn L, Finn SP, Cuffe S, Gray SG. Non-coding RNA repertoires in malignant pleural mesothelioma. Lung cancer (Amsterdam, Netherlands) **2015**;90(3):417-26 doi 10.1016/j.lungcan.2015.11.002.
37. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory Mechanisms and Intercellular Transfer of MicroRNAs in Living Cells. J Biol Chem **2010**;285(23):17442-52 doi 10.1074/jbc.M110.107821.
38. Li XQ, Liu JT, Fan LL, Liu Y, Cheng L, Wang F*, et al.* Exosomes derived from gefitinib-treated EGFR-mutant lung cancer cells alter cisplatin sensitivity via up-regulating autophagy. Oncotarget **2016**;7(17):24585-95 doi 10.18632/oncotarget.8358.
39. Marleau AM, Chen C-S, Joyce JA, Tullis RH. Exosome removal as a therapeutic adjuvant in cancer. Journal of translational medicine **2012**;10:134- doi 10.1186/1479-5876-10-134.
40. Safaei R, Larson BJ, Cheng TC, Gibson MA, Otani S, Naerdemann W*, et al.* Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. Molecular cancer therapeutics **2005**;4(10):1595-604 doi 10.1158/1535-7163.mct-05-0102.

41. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. Cancer research **2007**;67(7):2912-5 doi 10.1158/0008-5472.can-07-0520.
42. Iero M, Valenti R, Huber V, Filipazzi P, Parmiani G, Fais S*, et al.* Tumour-released exosomes and their implications in cancer immunity. Cell death and differentiation **2008**;15(1):80-8 doi 10.1038/sj.cdd.4402237.
43. Webber J, Steadman R, Mason MD, Tabi Z, Clayton A. Cancer exosomes trigger fibroblast to myofibroblast differentiation. Cancer research **2010**;70(23):9621-30 doi 10.1158/0008-5472.CAN-10-1722.
44. Zhang HG, Grizzle WE. Exosomes and cancer: a newly described pathway of immune suppression. Clinical cancer research : an official journal of the American Association for Cancer Research **2011**;17(5):959-64 doi 10.1158/1078-0432.ccr-10-1489.
45. Kosaka N, Yoshioka Y, Tominaga N, Hagiwara K, Katsuda T, Ochiya T. Dark side of the exosome: the role of the exosome in cancer metastasis and targeting the exosome as a strategy for cancer therapy. Future oncology (London, England) **2014**;10(4):671-81 doi 10.2217/fon.13.222.
46. Adorno-Cruz V, Kibria G, Liu X, Doherty M, Junk DJ, Guan D*, et al.* Cancer stem cells: targeting the roots of cancer, seeds of metastasis, and sources of therapy resistance. Cancer research **2015**;75(6):924-9 doi 10.1158/0008-5472.can-14-3225.
47. Zhao L, Liu W, Xiao J, Cao B. The role of exosomes and "exosomal shuttle microRNA" in tumorigenesis and drug resistance. Cancer letters **2015**;356(2 Pt B):339-46 doi 10.1016/j.canlet.2014.10.027.
48. van Zandwijk N, Pavlakis N, Kao SC, Linton A, Boyer MJ, Clarke S*, et al.* Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. The Lancet Oncology **2017**;18(10):1386-96 doi 10.1016/s1470-2045(17)30621-6.
49. Mukherjee K, Ghoshal B, Ghosh S, Chakrabarty Y, Shwetha S, Das S*, et al.* Reversible HuR-microRNA binding controls extracellular export of miR-122 and augments stress response. EMBO Rep **2016**;17(8):1184-203 doi 10.15252/embr.201541930.
50. Guo M, Wu F, Hu G, Chen L, Xu J, Xu P*, et al.* Autologous tumor cell-derived microparticle-based targeted chemotherapy in lung cancer patients with malignant pleural effusion. Science translational medicine **2019**;11(474) doi 10.1126/scitranslmed.aat5690.

CHAPTER FIVE: DISCUSSION AND FUTURE DIRECTIONS

The work conducted within this thesis was the culmination of nearly five years of work based upon the novel ideas generated within the Shukla lab. The genesis of these research studies, which thus far have resulted in three research publications, were an effort to fill parts of the enormous knowledge gap in the field of asbestos induced mesothelioma. Specifically, the projects and data enumerated upon above were works to uncover the layers of two dominant themes of cellular secretion and communication: how does asbestos inhalation in the lung lead to disease on distant mesothelial cells, and how mesothelioma cancer cells' biology is regulated by secreted factors in exosomes. These two concepts (or questions) were coupled with the hopes of potentially finding biomarkers of asbestos exposure or MM, and to identify possible therapeutic targets. The focal plane of this exciting work was homed in on the secretory vesicles known as exosomes, and we proudly made multiple first-of-their-kind discoveries regarding exosomes and MM.

Our initial investigations were to assay the proteomic content of exosomes secreted from asbestos exposed cells, and assess if these exosomes could potentiate changes to mesothelial cells that might mimic tumor-like transitions. Within this context, we employed both in vitro and in vivo experiments.

The in vivo study resulted in a descriptive set of evidence on serum exosomes' proteomic signature in mice after asbestos exposure. We exposed mice to asbestos via oropharyngeal aspiration and after 56 days harvested the serum exosomes for proteomic analysis. Tandem mass tag labeling and subsequent mass spectrometry provided data that differential

abundances of certain proteins were measurable in asbestos exposed mice serum exosomes as compared to controls. We focused on those proteins increased in abundance as our research is looking for potential biomarker identification. The increased exosomal proteins which were validated by Western blot analysis were ceruloplasmin, haptoglobin, and fibulin-1. In support of our findings these proteins have been identified before by other groups in response to asbestos exposure or mesothelioma, however, not in exosomes. Additionally, we identified 376 total exosomal proteins between both groups. The main finding here was that we were capable of identifying a proteomic signature based on abundance differences in serum exosomes between control and asbestos exposed mice. This demonstrates that asbestos exposure, at an organismal wide level, can lead to systemic changes in circulating exosomal protein content. The fact that we can measure these differences is an important step forward in the exosome field of asbestos exposure.

One of our core concepts about this work was to explore if exosomes secreted from asbestos exposed lung epithelial cells and macrophages can affect mesothelial cells. Our in vitro work on asbestos exposure attempted to answer this question. First, we identified protein dissimilarities in exosomes from asbestos exposed cells, and then studied target cell effects on mesothelial cells. Using two experimental set-ups, both epithelial cells and macrophages, as these are the first cells in the lung to encounter asbestos fibers; we exposed each cell type to asbestos and collected their exosomes.

Proteomic analysis of epithelial cells indicated significant differential abundances of 145 proteins, including increased abundance in the asbestos group: plasminogen activator inhibitor 1, vimentin, 14-3-3 protein sigma, thrombospondin, transitional endoplasmic

reticulum ATPase, and glypican-1. Western blot validation was performed for glypican-1, notable for its role as an exosomal biomarker for pancreatic cancer, and thrombospondin which is overexpressed in MM tumors. Furthermore, exosomes from these asbestos exposed epithelial cells altered the gene expression of EMT related genes E-cadherin, desmoplakin, and IL1 Receptor Antagonist (IL1RN) in target mesothelial cells, potentiating the cells to becoming more mesenchymal, a hallmark of tumorigenesis.

The same experimental setup was then performed on macrophages (with the exception of the means of gene expression analysis, microarray instead of PCR array, and the incorporation of an asbestos fiber exposed positive control group of mesothelial cells). Asbestos exposed macrophages secreted exosomes with 32 differentially abundant proteins compared to control cells' exosomes. Two were validated by Western blot, superoxide dismutase, elevated in asbestos exposure models described elsewhere and in mesothelioma studies elsewhere, and vimentin, a key regulator in the response to asbestos exposure by regulating the NLRP3 inflammasome.

Targeting mesothelial cells with exosomes from asbestos exposed macrophages led to gene expression modulation in 498 genes , 241 up and 257 down, and importantly, 206 of these genes were changed when mesothelial cells were exposed to asbestos fibers alone. This told us very plainly that exosomes from asbestos exposed cells can alter mesothelial cells in some of the same ways that asbestos fibers alone do. Especially because, after validation of six of these genes (upregulated *hCCNB2*, *hEGR1* and *hFANCD2*, and downregulated *hCRELD2*, *hERO1B* and *hJAG1*), we connected the expression differences to be of potential significance in mesothelial cells becoming more tumorigenic in capacity.

The two studies mentioned above have a few major takeaways: Firstly, asbestos exposure leads to measurable protein differences in exosomes both from cells in vitro and in serum of whole mice and after further validation can be used as biomarkers of asbestos exposure for the early diagnosis of mesothelioma. Secondly, we were able to prove that exosomes from lung epithelial cells and macrophages can alter the gene expression of target mesothelial cells in ways that may be inducing the necessary changes that beget MM tumor development.

We also unsuccessfully performed some in vivo experiments where mice were inoculated with MM tumors and 24 h later were injected with exosomes from the same tumor cell line. The intent was to show the effect of exosomes on tumor growth and metastasis. We did not observe any effect of tumor exosomes on tumor weight/volume or metastasis in this preliminary 4-week experiment.

Our next steps for these studies are to perform our in vivo work with larger groups of mice to increase statistical power, and study the exosomal proteomic signature of human serum of healthy patients, asbestos exposed patients and patients with MM after asbestos exposure. In addition, we want to recapitulate the targeting effects of asbestos-induced lung exosomes on mesothelial cells in vivo.

Shifting gears to the final study of this thesis, we indulged in the focus of exosome-contained miRNAs from MM tumor cells themselves. This study provided insight into the miRNA composition of MM tumor exosomes, shedding light on biological information that may be used as possible biomarkers for MM in the future. The initial hypothesis, along with the intent of characterization of MM exosomal miRNAs, went along the common

dogma of tumor exosomes that MM tumor cells secrete high volumes of exosomes with pro-tumorigenic miRNAs that allow signaling in the tumor microenvironment to aid MM growth and invasion. It turns out we were completely mistaken.

The MM tumor cells we cultured all secreted exosomes with upregulated levels of tumor suppressor miRNAs, particularly one that is of known importance in MM, miR-16-5p. This miRNA is well known to be underexpressed in MM tumor cells and tumor specimens. The function of miR-16-5p is that it prevents expression of oncogenic proteins, particularly BCL2 and CCND1. Because we saw increased secretion of tumor suppressor miRNAs such as miR-16-5p, we altered the hypothesis to state that MM tumor cells secrete high levels of tumor suppressor miRNAs via exosomes to rid themselves of the anti-tumorigenic effects.

We first employed small molecule inhibitors to inhibit exosomes secretion and found that upon reducing secretion of exosomes, we saw concomitant reductions in miR-16-5p secretion. This reduced secretion correlated with two major findings: miR-16-5p was indeed being secreted from MM tumor cells through the exosome pathway, and blocking secretion led to direct increases in the intracellular stores of miR-16-5p.

Exosome secretion inhibition led to a variety of anti-tumorigenic effects also: reduced proliferation, increased efficacy of cisplatin chemotherapeutics, reduced 3D spheroid growth, reduced colony formation, increased apoptosis, and reduced migration and invasion. This showed that exosome secretion may be a potential target in MM therapy, and that chemotherapeutic resistance may be in part related to exosome secretion. Furthermore, exosome inhibition led to significantly reduced amounts of miR-16-5p target

oncoproteins BCL2 and CCND1, telling us that the effects seen were at least in part dependent on miR-16-5p being restored.

The following experiment was to force-feed concentrated MM tumor exosomes back to the producer tumor cells. Upon this force-feeding, we witnessed significant levels of cell death in multiple MM subtypes. This was also correlated with BCL2 and CCND1 being reduced in the force-fed tumor cells indicating that the exosomal miR-16-5p was playing a role. Additionally, MM tumor exosomes did not kill healthy mesothelial cells, nor did healthy mesothelial cell exosomes have any killing effect on either cancer or non-cancer cells. This indicates that force-feeding may also be a new approach to MM therapeutics and its effects appear to be tumor cell specific.

To verify that these results were miR-16 specific, we directly transfected tumor cells with miR-16-5p mimic. The results from exosome inhibition and force-feeding were recapitulated, providing further evidence of the role miR-16-5p is playing in this approach. However, we did see something contradicting in our results; miR-16-5p transfection led to increased miR-16-5p intracellularly, as expected, but led to significantly less miR-16-5p in the exosomes.

Moreover, we were given a lead on to an RNA binding protein, known as HuR, that may also be involved in the mechanism of miR-16-5p going into exosomes. HuR has been shown to interact directly with miR-16-5p in the cytoplasm, so we though it may be a player in putting miR-16-5p into exosomes. Upon siRNA knockdown of HuR, we saw significant reduction in exosomal miR-16-5p and increased intracellular miR-16-5p, proving a role for HuR in miR-16-5p packaging. Further, we also found that HuR is directly

targeted by miR-16-5p, allowing for an interpretation to the contradictive evidence indicated in the previous paragraph.

This evidence allowed for the development of a schemed mechanism of action where HuR promotes miR-16-5p loading into exosomes, so siRNA knockdown of HuR leads to reduced exosomal miR-16-5p, and hence increased miR-16-5p in the cell. It is then clear that miR-16-5p targets HuR expression at the protein level, meaning that high miR-16-5p levels lead to low HuR and therefore, reduced exosomal miR-16-5p, which is exactly what our data represents. Because we also showed that miR-16-5p upregulation by transfection does not affect exosome secretion by particle number, we know that this effect is limited to the packaging process of exosome cargo. Along this logic, we theorize that MM cancer cells not only have the evolutionary advantage for uncontrolled cell growth because of low intracellular stores of miR-16-5p based on high exosomal removal, but that miR-16-5p itself negatively regulates its own packaging into exosomes by targeting HuR.

The next steps forward are to perform the exosome inhibition and force-feeding concepts in mouse models of MM to understand if they function similarly in in vivo settings. We also aim to conduct miRNA analysis of human serum exosomes from MM patients and asbestos exposed individuals compared to healthy people. Additionally, we find that because exosome targeting may be of use in cancer treatment, there may be a role of cancer prevention if exosome targeting can be employed specifically to cells that are contributing to the development of MM (as in asbestos exposed epithelial cells or macrophages in the lung). These future experiments will undoubtedly add to the already innovative and

important work described within this thesis, and it will be very exciting to see what results are yielded.

Altogether, this evidence provides novel insight into tumorigenic mechanisms that may be targeted in potential therapeutic advancements in MM. We have shown proteomic evidence of differentially abundant proteins in exosomes from asbestos exposure, both in vitro and in vivo. We have implicated asbestos exposed cells' exosomes as being messengers to mesothelial cells and thereby modulating them to having more tumorigenic gene expression. MM exosomes, based on this work, appear to be conduits of tumorigenic growth by means of removing the tumor suppressor miR-16-5p. This mode of ridding miR-16-5p can be targeted by exosome inhibition and force-feeding MM exosomes to MM cells resulting in tumor cell death and reduced tumorigenic capacities. Based on all this, there is reason to expect that future experiments will add to this promising outlook for potential therapy of MM.

COMPREHENSIVE BIBLIOGRAPHY

Adorno-Cruz, V., G. Kibria, X. Liu, M. Doherty, D.J. Junk, D. Guan, C. Hubert, M. Venere, E. Mulkearns-Hubert, M. Sinyuk, A. Alvarado, A.I. Caplan, J. Rich, S.L. Gerson, J. Lathia, and H. Liu. 2015. Cancer stem cells: targeting the roots of cancer, seeds of metastasis, and sources of therapy resistance. *Cancer research*. 75:924-929.

Adusumilli, P.S., L. Cherkassky, J. Villena-Vargas, C. Colovos, E. Servais, J. Plotkin, D.R. Jones, and M. Sadelain. 2014. Regional delivery of mesothelin-targeted CAR T cell therapy generates potent and long-lasting CD4-dependent tumor immunity. *Science translational medicine*. 6:261ra151.

Afanas'eva, I.S., V.A. Spitsyn, and G.V. Tsurikova. 1993. [Genetic polymorphism of haptoglobin and quantitative changes in its levels during exposure to asbestos]. *Genetika*. 29:1895-1900.

Al-Nedawi, K., B. Meehan, J. Micallef, V. Lhotak, L. May, A. Guha, and J. Rak. 2008. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nature cell biology*. 10:619-624.

Alegre, E., M. Sammamed, S. Fernandez-Landazuri, L. Zubiri, and A. Gonzalez. 2015. Circulating biomarkers in malignant melanoma. *Advances in clinical chemistry*. 69:47-89.

Andaloussi, E.L., I. Mager, X.O. Breakefield, and M.J. Wood. 2013. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nature reviews. Drug discovery*. 12:347-357.

Arntz, O.J., B.C. Pieters, M.C. Oliveira, M.G. Broeren, M.B. Bennink, M. de Vries, P.L. van Lent, M.I. Koenders, W.B. van den Berg, P.M. van der Kraan, and F.A. van de Loo. 2015. Oral administration of bovine milk-derived extracellular vesicles attenuates arthritis in two mouse models. *Molecular nutrition & food research*.

Bach, D.H., J.Y. Hong, H.J. Park, and S.K. Lee. 2017. The role of exosomes and miRNAs in drug-resistance of cancer cells. *International journal of cancer*. 141:220-230.

Bakker, E., A. Guazzelli, F. Ashtiani, C. Demonacos, M. Krstic-Demonacos, and L. Mutti. 2017. Immunotherapy advances for mesothelioma treatment. *Expert review of anticancer therapy*. 17:799-814.

Bard, M.P., J.P. Hegmans, A. Hemmes, T.M. Luider, R. Willemsen, L.A. Severijnen, J.P. van Meerbeeck, S.A. Burgers, H.C. Hoogsteden, and B.N. Lambrecht. 2004. Proteomic analysis of exosomes isolated from human malignant pleural effusions. *American journal of respiratory cell and molecular biology*. 31:114-121.

Beckett, P., J. Edwards, D. Fennell, R. Hubbard, I. Woolhouse, and M.D. Peake. 2015. Demographics, management and survival of patients with malignant pleural mesothelioma in the National Lung Cancer Audit in England and Wales. *Lung cancer (Amsterdam, Netherlands)*. 88:344-348.

Berman, D.W., and K.S. Crump. 2008. Update of potency factors for asbestos-related lung cancer and mesothelioma. *Critical reviews in toxicology*. 38 Suppl 1:1-47.

Bibby, A.C., S. Tsim, N. Kanellakis, H. Ball, D.C. Talbot, K.G. Blyth, N.A. Maskell, and I. Psallidas. 2016. Malignant pleural mesothelioma: an update on investigation, diagnosis and treatment. *European respiratory review : an official journal of the European Respiratory Society*. 25:472-486.

Birnie, K.A., C.M. Prêle, P.J. Thompson, B. Badrian, and S.E. Mutsaers. 2017. Targeting microRNA to improve diagnostic and therapeutic approaches for malignant mesothelioma. *Oncotarget*. 8:78193-78207.

Blanc, L., and M. Vidal. 2017. New insights into the function of Rab GTPases in the context of exosomal secretion. *Small GTPases*:1-12.

Bobrie, A., M. Colombo, G. Raposo, and C. Thery. 2011. Exosome secretion: molecular mechanisms and roles in immune responses. *Traffic (Copenhagen, Denmark)*. 12:1659-1668.

Bobrie, A., S. Krumeich, F. Reyal, C. Recchi, L.F. Moita, M.C. Seabra, M. Ostrowski, and C. Thery. 2012. Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression. *Cancer research*. 72:4920-4930.

Boulanger, C.M., X. Loyer, P.E. Rautou, and N. Amabile. 2017. Extracellular vesicles in coronary artery disease. *Nature reviews. Cardiology*. 14:259-272.

Braicu, C., C. Tomuleasa, P. Monroig, A. Cucuianu, I. Berindan-Neagoe, and G.A. Calin. 2015. Exosomes as divine messengers: are they the Hermes of modern molecular oncology? *Cell death and differentiation*. 22:34-45.

Brinton, L.T., H.S. Sloane, M. Kester, and K.A. Kelly. 2015. Formation and role of exosomes in cancer. *Cellular and molecular life sciences : CMLS*. 72:659-671.

Bruno, R., A.M. Poma, G. Alì, R. Giannini, G. Puppo, F. Melfi, M. Lucchi, A. Mussi, A. Falcone, A. Chella, and G. Fontanini. 2017. Novel prognostic markers for epithelioid malignant pleural mesothelioma. *Journal of Clinical Oncology*. 35:e20028-e20028.

Buschow, S.I., B.W. van Balkom, M. Aalberts, A.J. Heck, M. Wauben, and W. Stoorvogel. 2010. MHC class II-associated proteins in B-cell exosomes and potential functional implications for exosome biogenesis. *Immunology and cell biology*. 88:851-856.

Campion, C.G., O. Sanchez-Ferras, and S.N. Batchu. 2017. Potential Role of Serum and Urinary Biomarkers in Diagnosis and Prognosis of Diabetic Nephropathy. *Canadian journal of kidney health and disease*. 4:2054358117705371.

Carbone, M., H.I. Pass, P. Rizzo, M. Marinetti, M. Di Muzio, D.J. Mew, A.S. Levine, and A. Procopio. 1994. Simian virus 40-like DNA sequences in human pleural mesothelioma. *Oncogene*. 9:1781-1790.

Casarsa, C., N. Bassani, F. Ambrogi, G. Zabucchi, P. Boracchi, E. Biganzoli, and D. Coradini. 2011. Epithelial-to-mesenchymal transition, cell polarity and stemness-associated features in malignant pleural mesothelioma. *Cancer letters*. 302:136-143.

Cavalleri, T., L. Angelici, C. Favero, L. Dioni, C. Mensi, C. Bareggi, A. Palleschi, A. Rimessi, D. Consonni, L. Bordini, A. Todaro, V. Bollati, and A.C. Pesatori. 2017. Plasmatic extracellular vesicle microRNAs in malignant pleural mesothelioma and asbestos-exposed subjects suggest a 2-miRNA signature as potential biomarker of disease. *PLoS ONE*. 12:e0176680.

Cha, D.J., J.L. Franklin, Y. Dou, Q. Liu, J.N. Higginbotham, M. Demory Beckler, A.M. Weaver, K. Vickers, N. Prasad, S. Levy, B. Zhang, R.J. Coffey, and J.G. Patton. 2015. KRAS-dependent sorting of miRNA to exosomes. *eLife*. 4.

Chalmin, F., S. Ladoire, G. Mignot, J. Vincent, M. Bruchard, J.P. Remy-Martin, W. Boireau, A. Rouleau, B. Simon, D. Lanneau, A. De Thonel, G. Multhoff, A. Hamman, F. Martin, B. Chauffert, E. Solary, L. Zitvogel, C. Garrido, B. Ryffel, C. Borg, L. Apetoh, C. Rebe, and F. Ghiringhelli. 2010. Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *J Clin Invest*. 120:457-471.

Chaput, N., and C. Thery. 2011. Exosomes: immune properties and potential clinical implementations. *Seminars in immunopathology*. 33:419-440.

Chargaff, E., and R. West. 1946. The biological significance of the thromboplastic protein of blood. *J Biol Chem*. 166:189-197.

Chen, K., and N. Rajewsky. 2007. The evolution of gene regulation by transcription factors and microRNAs. *Nature reviews. Genetics*. 8:93-103.

Chen, W.X., X.M. Liu, M.M. Lv, L. Chen, J.H. Zhao, S.L. Zhong, M.H. Ji, Q. Hu, Z. Luo, J.Z. Wu, and J.H. Tang. 2014. Exosomes from drug-resistant breast cancer cells transmit chemoresistance by a horizontal transfer of microRNAs. *PLoS One*. 9:e95240.

Cheruvanky, A., H. Zhou, T. Pisitkun, J.B. Kopp, M.A. Knepper, P.S.T. Yuen, and R.A. Star. 2007. Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. *American journal of physiology. Renal physiology*. 292:F1657-F1661.

Chevillet, J.R., Q. Kang, I.K. Ruf, H.A. Briggs, L.N. Vojtech, S.M. Hughes, H.H. Cheng, J.D. Arroyo, E.K. Meredith, E.N. Gallichotte, E.L. Pogosova-Agadjanyan, C. Morrissey, D.L. Stirewalt, F. Hladik, E.Y. Yu, C.S. Higano, and M. Tewari. 2014. Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proceedings of the National Academy of Sciences of the United States of America*. 111:14888-14893.

Chiam, K., T. Wang, D.I. Watson, G.C. Mayne, T.S. Irvine, T. Bright, L. Smith, I.A. White, J.M. Bowen, D. Keefe, S.K. Thompson, M.E. Jones, and D.J. Hussey. 2015. Circulating Serum Exosomal miRNAs As Potential Biomarkers for Esophageal Adenocarcinoma. *Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract*. 19:1208-1215.

Ching, R.C., and P.J. Kingham. 2015. The role of exosomes in peripheral nerve regeneration. *Neural regeneration research*. 10:743-747.

Chiou, N.-T., and K.M. Ansel. 2016. Improved exosome isolation by sucrose gradient fractionation of ultracentrifuged crude exosome pellets.

Cimmino, A., G.A. Calin, M. Fabbri, M.V. Iorio, M. Ferracin, M. Shimizu, S.E. Wojcik, R.I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C.G. Liu, T.J. Kipps, M. Negrini, and C.M. Croce. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proceedings of the National Academy of Sciences of the United States of America*. 102:13944-13949.

Clayton, A., and Z. Tabi. 2005. Exosomes and the MICA-NKG2D system in cancer. *Blood cells, molecules & diseases*. 34:206-213.

Colletti, M., A. Petretto, A. Galardi, V.D. Paolo, L. Tomao, C. Lavarello, E. Inglese, M. Bruschi, A.A. Lopez, L. Pascucci, B. Geoerger, H. Peinado, L. Franco, and D.G. Angela. 2017. Proteomic analysis of neuroblastoma-derived exosomes: new insights into a metastatic signature. *Proteomics*.

Colombo, M., C. Moita, G. van Niel, J. Kowal, J. Vigneron, P. Benaroch, N. Manel, L.F. Moita, C. Thery, and G. Raposo. 2013. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *Journal of cell science*. 126:5553-5565.

Colombo, M., G. Raposo, and C. Thery. 2014. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annual review of cell and developmental biology*. 30:255-289.

Costa-Silva, B., N.M. Aiello, A.J. Ocean, S. Singh, H. Zhang, B.K. Thakur, A. Becker, A. Hoshino, M.T. Mark, H. Molina, J. Xiang, T. Zhang, T.M. Theilen, G. Garcia-Santos, C. Williams, Y. Ararso, Y. Huang, G. Rodrigues, T.L. Shen, K.J. Labori, I.M. Lothe, E.H. Kure, J. Hernandez,

A. Doussot, S.H. Ebbesen, P.M. Grandgenett, M.A. Hollingsworth, M. Jain, K. Mallya, S.K. Batra, W.R. Jarnagin, R.E. Schwartz, I. Matei, H. Peinado, B.Z. Stanger, J. Bromberg, and D. Lyden. 2015. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nature cell biology*. 17:816-826.

Coumans, F.A.W., A.R. Brisson, E.I. Buzas, F. Dignat-George, E.E.E. Drees, S. El-Andaloussi, C. Emanueli, A. Gasecka, A. Hendrix, A.F. Hill, R. Lacroix, Y. Lee, T.G. van Leeuwen, N. Mackman, I. Mager, J.P. Nolan, E. van der Pol, D.M. Pegtel, S. Sahoo, P.R.M. Siljander, G. Sturk, O. de Wever, and R. Nieuwland. 2017. Methodological Guidelines to Study Extracellular Vesicles. *Circulation research*. 120:1632-1648.

Craighead, J.E., and B.T. Mossman. 1982. The pathogenesis of asbestos-associated diseases. *N Engl J Med*. 306:1446-1455.

Craighead , J.E., and B.T. Mossman 1982. The Pathogenesis of Asbestos-Associated Diseases. *New England Journal of Medicine*. 306:1446-1455.

Creaney, J., I.M. Dick, J.S. Leon, and B.W. Robinson. 2017. A Proteomic Analysis of the Malignant Mesothelioma Secretome Using iTRAQ. *Cancer genomics & proteomics*. 14:103-117.

Creaney, J., I.M. Dick, T.M. Meniawy, S.L. Leong, J.S. Leon, Y. Demelker, A. Segal, A.W. Musk, Y.C. Lee, S.J. Skates, A.K. Nowak, and B.W. Robinson. 2014. Comparison of fibulin-3 and mesothelin as markers in malignant mesothelioma. *Thorax*. 69:895-902.

Crispi, S., R.A. Calogero, M. Santini, P. Mellone, B. Vincenzi, G. Citro, G. Vicidomini, S. Fasano, R. Meccariello, G. Cobellis, S. Menegozzo, R. Pierantoni, F. Facciolo, A. Baldi, and M. Menegozzo. 2009. Global Gene Expression Profiling Of Human Pleural Mesotheliomas: Identification of Matrix Metalloproteinase 14 (MMP-14) as Potential Tumour Target. *PLOS ONE*. 4:e7016.

Cuffe, J.S.M., O. Holland, C. Salomon, G.E. Rice, and A.V. Perkins. 2017. Review: Placental derived biomarkers of pregnancy disorders. *Placenta*. 54:104-110.

Currie, G.P., S.J. Watt, and N.A. Maskell. 2009. An overview of how asbestos exposure affects the lung. *BMJ*. 339:b3209.

Davalieva, K., and M. Polenakovic. 2015. Proteomics in diagnosis of prostate cancer. *Prilozi / Makedonska akademija na naukite i umetnostite, Oddelenie za bioloski i medicinski nauki = Contributions / Macedonian Academy of Sciences and Arts, Section of Biological and Medical Sciences*. 36:5-36.

Davidson, B. 2007. Expression of cancer-associated molecules in malignant mesothelioma. *Biomarker insights*. 2:173-184.

de Gassart, A., C. Geminard, B. Fevrier, G. Raposo, and M. Vidal. 2003. Lipid raft-associated protein sorting in exosomes. *Blood*. 102:4336-4344.

De Santi, C., O. Melaiu, A. Bonotti, L. Cascione, G. Di Leva, R. Foddis, A. Cristaudo, M. Lucchi, M. Mora, A. Truini, A. Tironi, B. Murer, R. Boldorini, M. Cipollini, F. Gemignani, P. Gasparini, L. Mutti, and S. Landi. 2017. Deregulation of miRNAs in malignant pleural mesothelioma is associated with prognosis and suggests an alteration of cell metabolism. *Sci Rep*. 7:3140.

Delage, E., D.C. Cervantes, E. Penard, C. Schmitt, S. Syan, A. Disanza, G. Scita, and C. Zurzolo. 2016. Differential identity of Filopodia and Tunneling Nanotubes revealed by the opposite functions of actin regulatory complexes. *Sci Rep*. 6:39632.

dos Santos, G., M.R. Rogel, M.A. Baker, J.R. Troken, D. Urich, L. Morales-Nebreda, J.A. Sennello, M.A. Kutuzov, A. Sitikov, J.M. Davis, A.P. Lam, P. Cheresh, D. Kamp, D.K. Shumaker, G.R.

Budinger, and K.M. Ridge. 2015. Vimentin regulates activation of the NLRP3 inflammasome. *Nature communications*. 6:6574.

Dostert, C., V. Petrilli, R. Van Bruggen, C. Steele, B.T. Mossman, and J. Tschopp. 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science (New York, N.Y.)*. 320:674-677.

Dovrat, S., M. Caspi, A. Zilberberg, L. Lahav, A. Firsow, H. Gur, and R. Rosin-Arbesfeld. 2014. 14-3-3 and β-catenin are secreted on extracellular vesicles to activate the oncogenic Wnt pathway. *Mol Oncol*. 8:894-911.

Dozier, J., H. Zheng, and P.S. Adusumilli. 2017. Immunotherapy for malignant pleural mesothelioma: current status and future directions. *Transl Lung Cancer Res*. 6:315-324.

Dragon, J., J. Thompson, M. MacPherson, and A. Shukla. 2015. Differential Susceptibility of Human Pleural and Peritoneal Mesothelial Cells to Asbestos Exposure. *Journal of cellular biochemistry*. 116:1540-1552.

Dutta, S., O. Reamtong, W. Pangvongsa, S. Kitdumrongthum, K. Janpipatkul, P. Sangvanich, P. Piyachaturawat, and A. Chairoungdua. 2015. Proteomics profiling of cholangiocarcinoma exosomes: A potential role of oncogenic protein transferring in cancer progression. *Biochimica et biophysica acta*.

Dutta, S., C. Warshall, C. Bandyopadhyay, D. Dutta, and B. Chandran. 2014. Interactions between Exosomes from Breast Cancer Cells and Primary Mammary Epithelial Cells Leads to Generation of Reactive Oxygen Species Which Induce DNA Damage Response, Stabilization of p53 and Autophagy in Epithelial Cells. *PLoS ONE*. 9:e97580.

Eitan, E., J. Green, M. Bodogai, N.A. Mode, R. Baek, M.M. Jorgensen, D.W. Freeman, K.W. Witwer, A.B. Zonderman, A. Biragyn, M.P. Mattson, N. Noren Hooten, and M.K. Evans. 2017. Age-Related Changes in Plasma Extracellular Vesicle Characteristics and Internalization by Leukocytes. *Sci Rep*. 7:1342.

Essandoh, K., L. Yang, X. Wang, W. Huang, D. Qin, J. Hao, Y. Wang, B. Zingarelli, T. Peng, and G.C. Fan. 2015. Blockade of exosome generation with GW4869 dampens the sepsis-induced inflammation and cardiac dysfunction. *Biochimica et biophysica acta*. 1852:2362-2371.

Fassina, A., R. Cappellesso, V. Guzzardo, L. Dalla Via, S. Piccolo, L. Ventura, and M. Fassan. 2012. Epithelial-mesenchymal transition in malignant mesothelioma. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*. 25:86-99.

Fennell, D. 2017. miR-16: expanding the range of molecular targets in mesothelioma. *The Lancet. Oncology*.

Flores, R.M. 2016a. The mesothelioma surgery shift. *The Journal of thoracic and cardiovascular surgery*. 151:485-486.

Flores, R.M. 2016b. Pleurectomy decortication for mesothelioma: The procedure of choice when possible. *The Journal of thoracic and cardiovascular surgery*. 151:310-312.

Frank, A.L., and T.K. Joshi. 2014. The global spread of asbestos. *Annals of global health*. 80:257-262.

Franko, A., Dodi, #x10D, M. -Fikfak, Arneri, #x107, N. , Dol, #x17E, and V. an. 2009. Manganese and Extracellular Superoxide Dismutase Polymorphisms and Risk for Asbestosis. *Journal of Biomedicine and Biotechnology*. 2009:6.

Friand, V., G. David, and P. Zimmermann. 2015. Syntenin and syndecan in the biogenesis of exosomes. *Biology of the cell / under the auspices of the European Cell Biology Organization*.

Frieden, E. 1971. Ceruloplasmin, A Link Between Copper and Iron Metabolism. *In* Bioinorganic Chemistry. Vol. 100. AMERICAN CHEMICAL SOCIETY. 292-321.

Gee, G.V., D.C. Koestler, B.C. Christensen, D.J. Sugarbaker, D. Ugolini, G.P. Ivaldi, M.B. Resnick, E.A. Houseman, K.T. Kelsey, and C.J. Marsit. 2010. Downregulated microRNAs in the differential diagnosis of malignant pleural mesothelioma. *International journal of cancer*. 127:2859-2869.

Gehrmann, U., S. Hiltbrunner, A.M. Georgoudaki, M.C. Karlsson, T.I. Naslund, and S. Gabrielsson. 2013. Synergistic induction of adaptive antitumor immunity by codelivery of antigen with alpha-galactosylceramide on exosomes. *Cancer research*. 73:3865-3876.

Ghio, A.J., J. Stonehuerner, J. Richards, and R.B. Devlin. 2008. Iron homeostasis in the lung following asbestos exposure. *Antioxidants & redox signaling*. 10:371-377.

Ginestra, A., M.D. La Placa, F. Saladino, D. Cassarà, H. Nagase, and M.L. Vittorelli. 1998. The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. *Anticancer Res*. 18:3433-3437.

Greening, D.W., S.K. Gopal, R.A. Mathias, L. Liu, J. Sheng, H.J. Zhu, and R.J. Simpson. 2015a. Emerging roles of exosomes during epithelial-mesenchymal transition and cancer progression. *Seminars in cell & developmental biology*. 40:60-71.

Greening, D.W., H. Ji, M. Chen, B.W. Robinson, I.M. Dick, J. Creaney, and R.J. Simpson. 2016. Secreted primary human malignant mesothelioma exosome signature reflects oncogenic cargo. *Sci Rep*. 6:32643.

Greening, D.W., R. Xu, H. Ji, B.J. Tauro, and R.J. Simpson. 2015b. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Methods in molecular biology (Clifton, N.J.)*. 1295:179-209.

Gross, J.C., V. Chaudhary, K. Bartscherer, and M. Boutros. 2012. Active Wnt proteins are secreted on exosomes. *Nature cell biology*. 14:1036-1045.

Gumireddy, K., A. Li, D.H. Chang, Q. Liu, A.V. Kossenkov, J. Yan, R.J. Korst, B.T. Nam, H. Xu, L. Zhang, G.A. Ganepola, L.C. Showe, and Q. Huang. 2015. AKAP4 is a circulating biomarker for non-small cell lung cancer. *Oncotarget*.

Guo, L., T. Zhang, Y. Xiong, and Y. Yang. 2015. Roles of NOTCH1 as a Therapeutic Target and a Biomarker for Lung Cancer: Controversies and Perspectives. *Disease markers*. 2015:520590.

Guo, M., F. Wu, G. Hu, L. Chen, J. Xu, P. Xu, X. Wang, Y. Li, S. Liu, S. Zhang, Q. Huang, J. Fan, Z. Lv, M. Zhou, L. Duan, T. Liao, G. Yang, K. Tang, B. Liu, X. Liao, X. Tao, and Y. Jin. 2019. Autologous tumor cell-derived microparticle-based targeted chemotherapy in lung cancer patients with malignant pleural effusion. *Science translational medicine*. 11.

Gyorgy, B., M.E. Hung, X.O. Breakefield, and J.N. Leonard. 2015. Therapeutic applications of extracellular vesicles: clinical promise and open questions. *Annual review of pharmacology and toxicology*. 55:439-464.

Haney, M.J., N.L. Klyachko, Y. Zhao, R. Gupta, E.G. Plotnikova, Z. He, T. Patel, A. Piroyan, M. Sokolsky, A.V. Kabanov, and E.V. Batrakova. 2015. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *Journal of controlled release : official journal of the Controlled Release Society*. 207:18-30.

Harischandra, D.S., S. Ghaisas, D. Rokad, and A.G. Kanthasamy. 2017. Exosomes in Toxicology: Relevance to Chemical Exposure and Pathogenesis of Environmentally Linked Diseases. *Toxicological sciences : an official journal of the Society of Toxicology*.

Hassan, R., S. Bullock, A. Premkumar, R.J. Kreitman, H. Kindler, M.C. Willingham, and I. Pastan. 2007. Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin given as a bolus I.V. infusion to patients with mesothelin-expressing mesothelioma, ovarian, and pancreatic cancers. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 13:5144-5149.

Hassan, R., E. Sharon, A. Thomas, J. Zhang, A. Ling, M. Miettinen, R.J. Kreitman, S.M. Steinberg, K. Hollevoet, and I. Pastan. 2014. Phase 1 study of the antimesothelin immunotoxin SS1P in combination with pemetrexed and cisplatin for front-line therapy of pleural mesothelioma and correlation of tumor response with serum mesothelin, megakaryocyte potentiating factor, and cancer antigen 125. *Cancer*. 120:3311-3319.

Hegmans, J.P., M.P. Bard, A. Hemmes, T.M. Luider, M.J. Kleijmeer, J.B. Prins, L. Zitvogel, S.A. Burgers, H.C. Hoogsteden, and B.N. Lambrecht. 2004. Proteomic analysis of exosomes secreted by human mesothelioma cells. *The American journal of pathology*. 164:1807-1815.

Helwa, I., J. Cai, M.D. Drewry, A. Zimmerman, M.B. Dinkins, M.L. Khaled, M. Seremwe, W.M. Dismuke, E. Bieberich, W.D. Stamer, M.W. Hamrick, and Y. Liu. 2017. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLoS One*. 12:e0170628.

Heusermann, W., J. Hean, D. Trojer, E. Steib, S. von Bueren, A. Graff-Meyer, C. Genoud, K. Martin, N. Pizzato, J. Voshol, D.V. Morrissey, S.E.L. Andaloussi, M.J. Wood, and N.C. Meisner-Kober. 2016. Exosomes surf on filopodia to enter cells at endocytic hot spots, traffic within endosomes, and are targeted to the ER. *The Journal of cell biology*. 213:173-184.

Hillegass, J.M., S.R. Blumen, K. Cheng, M.B. MacPherson, V. Alexeeva, S.A. Lathrop, S.L. Beuschel, J.L. Steinbacher, K.J. Butnor, M.E. Ramos-Nino, A. Shukla, T.A. James, D.J. Weiss, D.J. Taatjes, H.I. Pass, M. Carbone, C.C. Landry, and B.T. Mossman. 2011. Increased efficacy of doxorubicin delivered in multifunctional microparticles for mesothelioma therapy. *International journal of cancer*. 129:233-244.

Hillegass, J.M., J.M. Miller, M.B. MacPherson, C.M. Westbom, M. Sayan, J.K. Thompson, S.L. Macura, T.N. Perkins, S.L. Beuschel, V. Alexeeva, H.I. Pass, C. Steele, B.T. Mossman, and A. Shukla. 2013. Asbestos and erionite prime and activate the NLRP3 inflammasome that stimulates autocrine cytokine release in human mesothelial cells. *Part Fibre Toxicol*. 10:39.

Hillerdal, G. 1984. Asbestos related pleuropulmonary lesions and the erythrocyte sedimentation rate. *Thorax*. 39:752-758.

Hirvonen, A., J. Tuimala, T. Ollikainen, K. Linnainmaa, and V. Kinnula. 2002. Manganese superoxide dismutase genotypes and asbestos-associated pulmonary disorders. *Cancer letters*. 178:71-74.

Hoen, E.N.M.N.-t., E.J. van der Vlist, M. Aalberts, H.C.H. Mertens, B.J. Bosch, W. Bartelink, E. Mastrobattista, E.V.B. van Gaal, W. Stoorvogel, G.J.A. Arkesteijn, and M.H.M. Wauben. 2012. Quantitative and qualitative flow cytometric analysis of nanosized cell-derived membrane vesicles. *Nanomedicine: Nanotechnology, Biology and Medicine*. 8:712-720.

Hornick, N.I., J. Huan, B. Doron, N.A. Goloviznina, J. Lapidus, B.H. Chang, and P. Kurre. 2015. Serum Exosome MicroRNA as a Minimally-Invasive Early Biomarker of AML. *Sci Rep*. 5:11295.

Hristova, M., C. Veith, A. Habibovic, Y.W. Lam, B. Deng, M. Geiszt, Y.M. Janssen-Heininger, and A. van der Vliet. 2014. Identification of DUOX1-dependent redox signaling through protein S-glutathionylation in airway epithelial cells. *Redox biology*. 2:436-446.

Hung, M.E., and J.N. Leonard. 2015. Stabilization of exosome-targeting peptides via engineered glycosylation. *J Biol Chem*. 290:8166-8172.

Husain, A.N., T.V. Colby, N.G. Ordonez, T.C. Allen, R.L. Attanoos, M.B. Beasley, K.J. Butnor, L.R. Chirieac, A.M. Churg, S. Dacic, F. Galateau-Salle, A. Gibbs, A.M. Gown, T. Krausz, L.A. Litzky, A. Marchevsky, A.G. Nicholson, V.L. Roggli, A.K. Sharma, W.D. Travis, A.E. Walts, and M.R. Wick. 2018. Guidelines for Pathologic Diagnosis of Malignant Mesothelioma 2017 Update of the Consensus Statement From the International Mesothelioma Interest Group. *Archives of pathology & laboratory medicine*. 142:89-108.

Hylebos, M., G. Van Camp, J.P. van Meerbeeck, and K. Op de Beeck. 2016. The Genetic Landscape of Malignant Pleural Mesothelioma: Results from Massively Parallel Sequencing. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer*. 11:1615-1626.

IARC, W. 2012. Asbestos (chrysotile, amosite, crocidolite, tremolite, actinolite, and anthophyllite). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans A Review of Human Carcinogens;*. 100C:219-309.

Ibrahim, A.G., K. Cheng, and E. Marban. 2014. Exosomes as critical agents of cardiac regeneration triggered by cell therapy. *Stem cell reports*. 2:606-619.

Iero, M., R. Valenti, V. Huber, P. Filipazzi, G. Parmiani, S. Fais, and L. Rivoltini. 2008. Tumour-released exosomes and their implications in cancer immunity. *Cell death and differentiation*. 15:80-88.

Irion, U., and D. St Johnston. 2007. bicoid RNA localization requires specific binding of an endosomal sorting complex. *Nature*. 445:554-558.

Isola, A.L., and S. Chen. 2017. Exosomes: The Messengers of Health and Disease. *Current neuropharmacology*. 15:157-165.

Janas, T., M.M. Janas, K. Sapon, and T. Janas. 2015. Mechanisms of RNA loading into exosomes. *FEBS letters*. 589:1391-1398.

Ji, R., B. Zhang, X. Zhang, J. Xue, X. Yuan, Y. Yan, M. Wang, W. Zhu, H. Qian, and W. Xu. 2015. Exosomes derived from human mesenchymal stem cells confer drug resistance in gastric cancer. *Cell cycle (Georgetown, Tex.)*:0.

Johnsen, K.B., J.M. Gudbergsson, M.N. Skov, L. Pilgaard, T. Moos, and M. Duroux. 2014. A comprehensive overview of exosomes as drug delivery vehicles - endogenous nanocarriers for targeted cancer therapy. *Biochimica et biophysica acta*. 1846:75-87.

Joyce, J.A., and J.W. Pollard. 2009. Microenvironmental regulation of metastasis. *Nature reviews. Cancer*. 9:239-252.

Kahlert, C., S.A. Melo, A. Protopopov, J. Tang, S. Seth, M. Koch, J. Zhang, J. Weitz, L. Chin, A. Futreal, and R. Kalluri. 2014. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. *J Biol Chem*. 289:3869-3875.

Kahlos, K., P. Pääkkö, E. Kurttila, Y. Soini, and V.L. Kinnula. 2000. Manganese superoxide dismutase as a diagnostic marker for malignant pleural mesothelioma. *British Journal of Cancer*. 82:1022-1029.

Kalluri, R., and R.A. Weinberg. 2009. The basics of epithelial-mesenchymal transition. *The Journal of Clinical Investigation*. 119:1420-1428.

Kamp, D.W., P. Graceffa, W.A. Pryor, and S.A. Weitzman. 1992. The role of free radicals in asbestos-induced diseases. *Free radical biology & medicine*. 12:293-315.

Kamp, D.W., G. Liu, P. Cheresh, S.J. Kim, A. Mueller, A.P. Lam, H. Trejo, D. Williams, S. Tulasiram, M. Baker, K. Ridge, N.S. Chandel, and R. Beri. 2013. Asbestos-induced alveolar epithelial cell apoptosis. The role of endoplasmic reticulum stress response. *American journal of respiratory cell and molecular biology*. 49:892-901.

Kanlikilicer, P., M.H. Rashed, R. Bayraktar, R. Mitra, C. Ivan, B. Aslan, X. Zhang, J. Filant, A.M. Silva, C. Rodriguez-Aguayo, E. Bayraktar, M. Pichler, B. Ozpolat, G.A. Calin, A.K. Sood, and G. Lopez-Berestein. 2016. Ubiquitous Release of Exosomal Tumor Suppressor miR-6126 from Ovarian Cancer Cells. *Cancer research*. 76:7194-7207.

Kao, S.C., Y.Y. Cheng, M. Williams, M.B. Kirschner, J. Madore, T. Lum, K.H. Sarun, A. Linton, B. McCaughan, S. Klebe, N. van Zandwijk, R.A. Scolyer, M.J. Boyer, W.A. Cooper, and G. Reid. 2017. Tumor Suppressor microRNAs Contribute to the Regulation of PD-L1 Expression in Malignant Pleural Mesothelioma. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer*. 12:1421-1433.

Kaplan, A., M. Bueno, and A.E. Fournier. 2017. Extracellular functions of 14-3-3 adaptor proteins. *Cellular signalling*. 31:26-30.

Katakowski, M., B. Buller, X. Zheng, Y. Lu, T. Rogers, O. Osobamiro, W. Shu, F. Jiang, and M. Chopp. 2013. Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer letters*. 335:201-204.

Ke, F., J. Zhang, and S. Jin. 1991. [The relationship of biochemical changes among bronchoalveolar lavage fluid serum and lung on dust-exposed rats]. *Hua xi yi ke da xue xue bao = Journal of West China University of Medical Sciences = Huaxi yike daxue xuebao*. 22:292-295.

Keller, S., M.P. Sanderson, A. Stoeck, and P. Altevogt. 2006. Exosomes: from biogenesis and secretion to biological function. *Immunology letters*. 107:102-108.

Kim, M.C., F.J. Cui, and Y. Kim. 2013. Hydrogen peroxide promotes epithelial to mesenchymal transition and stemness in human malignant mesothelioma cells. *Asian Pacific journal of cancer prevention : APJCP*. 14:3625-3630.

Kirschner, M.B., E. Pulford, M.A. Hoda, A. Rozsas, K. Griggs, Y.Y. Cheng, J.J. Edelman, S.C. Kao, R. Hyland, Y. Dong, V. Laszlo, T. Klikovits, M.P. Vallely, M. Grusch, B. Hegedus, B. Dome, W. Klepetko, N. van Zandwijk, S. Klebe, and G. Reid. 2015. Fibulin-3 levels in malignant pleural mesothelioma are associated with prognosis but not diagnosis. *Br J Cancer*. 113:963-969.

Kobayashi, N., G. Kostka, J.H. Garbe, D.R. Keene, H.P. Bachinger, F.G. Hanisch, D. Markova, T. Tsuda, R. Timpl, M.L. Chu, and T. Sasaki. 2007. A comparative analysis of the fibulin protein family. Biochemical characterization, binding interactions, and tissue localization. *J Biol Chem*. 282:11805-11816.

Koppers-Lalic, D., M.M. Hogenboom, J.M. Middeldorp, and D.M. Pegtel. 2013. Virus-modified exosomes for targeted RNA delivery; A new approach in nanomedicine. *Advanced Drug Delivery Reviews*. 65:348-356.

Kosaka, N., H. Iguchi, Y. Yoshioka, F. Takeshita, Y. Matsuki, and T. Ochiya. 2010. Secretory Mechanisms and Intercellular Transfer of MicroRNAs in Living Cells. *J Biol Chem*. 285:17442-17452.

Kosaka, N., Y. Yoshioka, N. Tominaga, K. Hagiwara, T. Katsuda, and T. Ochiya. 2014. Dark side of the exosome: the role of the exosome in cancer metastasis and targeting the exosome as a strategy for cancer therapy. *Future oncology (London, England)*. 10:671-681.

Kosgodage, U.S., R.P. Trindade, P.R. Thompson, J.M. Inal, and S. Lange. 2017. Chloramidine/Bisindolylmaleimide-I-Mediated Inhibition of Exosome and Microvesicle Release and Enhanced Efficacy of Cancer Chemotherapy. *International journal of molecular sciences*. 18.

Kreimer, S., A.M. Belov, I. Ghiran, S.K. Murthy, D.A. Frank, and A.R. Ivanov. 2015. Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics. *Journal of proteome research*. 14:2367-2384.

Kumar, S. 2014. Bone defect repair in mice by mesenchymal stem cells. *Methods in molecular biology (Clifton, N.J.)*. 1213:193-207.

Lai, C.P., O. Mardini, M. Ericsson, S. Prabhakar, C.A. Maguire, J.W. Chen, B.A. Tannous, and X.O. Breakefield. 2014a. Dynamic biodistribution of extracellular vesicles in vivo using a multimodal imaging reporter. *ACS nano*. 8:483-494.

Lai, C.P., B.A. Tannous, and X.O. Breakefield. 2014b. Noninvasive in vivo monitoring of extracellular vesicles. *Methods in molecular biology (Clifton, N.J.)*. 1098:249-258.

Lamouille, S., J. Xu, and R. Derynck. 2014. Molecular mechanisms of epithelial–mesenchymal transition. *Nature reviews. Molecular cell biology*. 15:178-196.

Lane, R.E., D. Korbie, W. Anderson, R. Vaidyanathan, and M. Trau. 2015. Analysis of exosome purification methods using a model liposome system and tunable-resistive pulse sensing. *Sci Rep*. 5:7639.

Lazar, I., E. Clement, M. Ducoux-Petit, L. Denat, V. Soldan, S. Dauvillier, S. Balor, O. Burlet-Schiltz, L. Larue, C. Muller, and L. Nieto. 2015. Proteome characterization of melanoma exosomes reveals a specific signature for metastatic cell lines. *Pigment cell & melanoma research*. 28:464-475.

Lazarus, A.A., and A. Philip. 2011. Asbestosis. *Disease-a-month : DM*. 57:14-26.

Le, M.T.N., P. Hamar, C. Guo, E. Basar, Perdig, xE, R. o-Henriques, L. Balaj, and J. Lieberman. 2014. miR-200–containing extracellular vesicles promote breast cancer cell metastasis. *The Journal of Clinical Investigation*. 124:5109-5128.

Lee, E.Y., K.S. Park, Y.J. Yoon, J. Lee, H.G. Moon, S.C. Jang, K.H. Choi, Y.K. Kim, and Y.S. Gho. 2012a. Therapeutic effects of autologous tumor-derived nanovesicles on melanoma growth and metastasis. *PLoS One*. 7:e33330.

Lee, J.K., S.R. Park, B.K. Jung, Y.K. Jeon, Y.S. Lee, M.K. Kim, Y.G. Kim, J.Y. Jang, and C.W. Kim. 2013. Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS One*. 8:e84256.

Lee, Y., S. El Andaloussi, and M.J. Wood. 2012b. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Hum Mol Genet*. 21:R125-134.

Lee, Y.C., R.W. Light, and A.W. Musk. 2000. Management of malignant pleural mesothelioma: a critical review. *Current opinion in pulmonary medicine*. 6:267-274.

Li, M., M.E. Gunter, and N.K. Fukagawa. 2012. Differential activation of the inflammasome in THP-1 cells exposed to chrysotile asbestos and Libby "six-mix" amphiboles and subsequent activation of BEAS-2B cells. *Cytokine*. 60:718-730.

Li, P., M. Kaslan, S.H. Lee, J. Yao, and Z. Gao. 2017. Progress in Exosome Isolation Techniques. *Theranostics*. 7:789-804.

Li, X.Q., J.T. Liu, L.L. Fan, Y. Liu, L. Cheng, F. Wang, H.Q. Yu, J. Gao, W. Wei, H. Wang, and G.P. Sun. 2016. Exosomes derived from gefitinib-treated EGFR-mutant lung cancer cells alter cisplatin sensitivity via up-regulating autophagy. *Oncotarget*. 7:24585-24595.

Li, Y., Q. Zheng, C. Bao, S. Li, W. Guo, J. Zhao, D. Chen, J. Gu, X. He, and S. Huang. 2015. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell research*.

Lima, L.G., R. Chammas, R.Q. Monteiro, M.E. Moreira, and M.A. Barcinski. 2009. Tumor-derived microvesicles modulate the establishment of metastatic melanoma in a phosphatidylserine-dependent manner. *Cancer letters*. 283:168-175.

Linch, M., S. Gennatas, S. Kazikin, J. Iqbal, R. Gunapala, K. Priest, J. Severn, A. Norton, B. Ayite, J. Bhosle, M. O'Brien, and S. Popat. 2014. A serum mesothelin level is a prognostic indicator for patients with malignant mesothelioma in routine clinical practice. *BMC cancer*. 14:674-674.

Lo Cicero, A., P.D. Stahl, and G. Raposo. 2015. Extracellular vesicles shuffling intercellular messages: for good or for bad. *Current opinion in cell biology*. 35:69-77.

Lobb, R.J., R. van Amerongen, A. Wiegmans, S. Ham, J.E. Larsen, and A. Moller. 2017. Exosomes derived from mesenchymal non-small cell lung cancer cells promote chemoresistance. *International journal of cancer*. 141:614-620.

Lu, H., F. Niu, F. Liu, J. Gao, Y. Sun, and X. Zhao. 2017. Elevated glypican-1 expression is associated with an unfavorable prognosis in pancreatic ductal adenocarcinoma. *Cancer medicine*. 6:1181-1191.

Luan, X., K. Sansanaphongpricha, I. Myers, H. Chen, H. Yuan, and D. Sun. 2017. Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta pharmacologica Sinica*. 38:754-763.

Ludwig, A.K., and B. Giebel. 2012. Exosomes: small vesicles participating in intercellular communication. *The international journal of biochemistry & cell biology*. 44:11-15.

Lund, L.G., and A.E. Aust. 1991. Iron-catalyzed reactions may be responsible for the biochemical and biological effects of asbestos. *BioFactors (Oxford, England)*. 3:83-89.

MacLeod, N., A. Chalmers, N. O'Rourke, K. Moore, J. Sheridan, L. McMahon, C. Bray, J. Stobo, A. Price, M. Fallon, and B.J. Laird. 2015. Is Radiotherapy Useful for Treating Pain in Mesothelioma?: A Phase II Trial. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer*. 10:944-950.

Madison, M.N., and C.M. Okeoma. 2015. Exosomes: Implications in HIV-1 Pathogenesis. *Viruses*. 7:4093-4118.

Mahaweni, N.M., M.E. Kaijen-Lambers, J. Dekkers, J.G. Aerts, and J.P. Hegmans. 2013. Tumour-derived exosomes as antigen delivery carriers in dendritic cell-based immunotherapy for malignant mesothelioma. *Journal of extracellular vesicles*. 2.

Mahmoudi, K., A. Ezrin, and C. Hadjipanayis. 2015. Small extracellular vesicles as tumor biomarkers for glioblastoma. *Molecular aspects of medicine*.

Manning, C.B., V. Vallyathan, and B.T. Mossman. 2002. Diseases caused by asbestos: mechanisms of injury and disease development. *International immunopharmacology*. 2:191-200.

Markowitz, S.B., S.M. Levin, A. Miller, and A. Morabia. 2013. Asbestos, asbestosis, smoking, and lung cancer. New findings from the North American insulator cohort. *American journal of respiratory and critical care medicine*. 188:90-96.

Marleau, A.M., C.-S. Chen, J.A. Joyce, and R.H. Tullis. 2012. Exosome removal as a therapeutic adjuvant in cancer. *Journal of translational medicine*. 10:134-134.

Martinez, M.C., and R. Andriantsitohaina. 2017. Extracellular Vesicles in Metabolic Syndrome. *Circulation research*. 120:1674-1686.

Matsumura, T., K. Sugimachi, H. Iinuma, Y. Takahashi, J. Kurashige, G. Sawada, M. Ueda, R. Uchi, H. Ueo, Y. Takano, Y. Shinden, H. Eguchi, H. Yamamoto, Y. Doki, M. Mori, T. Ochiya, and K. Mimori. 2015. Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. *Br J Cancer*.

Mazurek, J.M., G. Syamlal, J.M. Wood, S.A. Hendricks, and A. Weston. 2017. Malignant Mesothelioma Mortality - United States, 1999-2015. *MMWR. Morbidity and mortality weekly report*. 66:214-218.

Melaiu, O., F. Gemignani, and S. Landi. 2018. The genetic susceptibility in the development of malignant pleural mesothelioma. *Journal of thoracic disease*. 10:S246-S252.

Melo, S.A., L.B. Luecke, C. Kahlert, A.F. Fernandez, S.T. Gammon, J. Kaye, V.S. LeBleu, E.A. Mittendorf, J. Weitz, N. Rahbari, C. Reissfelder, C. Pilarsky, M.F. Fraga, D. Piwnica-Worms, and R. Kalluri. 2015. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature*. 523:177-182.

Melo, S.A., H. Sugimoto, J.T. O'Connell, N. Kato, A. Villanueva, A. Vidal, L. Qiu, E. Vitkin, L.T. Perelman, C.A. Melo, A. Lucci, C. Ivan, G.A. Calin, and R. Kalluri. 2014. Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. *Cancer cell*. 26:707-721.

Mercer, R.R., A.F. Hubbs, J.F. Scabilloni, L. Wang, L.A. Battelli, S. Friend, V. Castranova, and D.W. Porter. 2011. Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes. *Part Fibre Toxicol*. 8:21.

Metintas, M., S. Metintas, G. Hillerdal, I. Ucgun, S. Erginel, F. Alatas, and H. Yildirim. 2005. Nonmalignant pleural lesions due to environmental exposure to asbestos: a field-based, cross-sectional study. *European Respiratory Journal*. 26:875-880.

Micolucci, L., M.M. Akhtar, F. Olivieri, M.R. Rippo, and A.D. Procopio. 2016. Diagnostic value of microRNAs in asbestos exposure and malignant mesothelioma: systematic review and qualitative meta-analysis. *Oncotarget*. 7:58606-58637.

Miller, I.V., and T.G. Grunewald. 2015. Tumour-derived exosomes: Tiny envelopes for big stories. *Biology of the cell / under the auspices of the European Cell Biology Organization*.

Millimaggi, D., M. Mari, S. D'Ascenzo, E. Carosa, E.A. Jannini, S. Zucker, G. Carta, A. Pavan, and V. Dolo. 2007. Tumor vesicle-associated CD147 modulates the angiogenic capability of endothelial cells. *Neoplasia (New York, N.Y.)*. 9:349-357.

Miserocchi, G., G. Sancini, F. Mantegazza, and G. Chiappino. 2008. Translocation pathways for inhaled asbestos fibers. *Environmental health : a global access science source*. 7:4-4.

Mitchell Cheung, C.W.M.a.J.R.T. 2017. Germline and somatic mutations in human mesothelioma and lessons from asbestos-exposed genetically engineered mouse models. *In* Asbestos and Mesothelioma. J.R. Testa, editor. Springer. 175-196.

Montecalvo, A., W.J. Shufesky, D.B. Stolz, M.G. Sullivan, Z. Wang, S.J. Divito, G.D. Papworth, S.C. Watkins, P.D. Robbins, A.T. Larregina, and A.E. Morelli. 2008. Exosomes as a short-range mechanism to spread alloantigen between dendritic cells during T cell allorecognition. *Journal of immunology (Baltimore, Md. : 1950)*. 180:3081-3090.

Moore, C., U. Kosgodage, S. Lange, and J.M. Inal. 2017. The emerging role of exosome and microvesicle- (EMV-) based cancer therapeutics and immunotherapy. *International journal of cancer*. 141:428-436.

Mossman, B.T. 1994. Carcinogenesis and related cell and tissue responses to asbestos: a review. *The Annals of occupational hygiene*. 38:617-624, 423.

Mossman, B.T., and A. Churg. 1998. Mechanisms in the pathogenesis of asbestosis and silicosis. *American journal of respiratory and critical care medicine*. 157:1666-1680.

Mossman, B.T., and J.B. Gee. 1989. Asbestos-related diseases. *N Engl J Med*. 320:1721-1730.

Mossman, B.T., and J.P. Marsh. 1989. Evidence supporting a role for active oxygen species in asbestos-induced toxicity and lung disease. *Environmental health perspectives*. 81:91-94.

Mossman, B.T., J.P. Marsh, and M.A. Shatos. 1986. Alteration of superoxide dismutase activity in tracheal epithelial cells by asbestos and inhibition of cytotoxicity by antioxidants. *Laboratory investigation; a journal of technical methods and pathology*. 54:204-212.

Mossman, B.T., A. Shukla, N.H. Heintz, C.F. Verschraegen, A. Thomas, and R. Hassan. 2013. New insights into understanding the mechanisms, pathogenesis, and management of malignant mesotheliomas. *The American journal of pathology*. 182:1065-1077.

Mukherjee, K., B. Ghoshal, S. Ghosh, Y. Chakrabarty, S. Shwetha, S. Das, and S.N. Bhattacharyya. 2016. Reversible HuR-microRNA binding controls extracellular export of miR-122 and augments stress response. *EMBO Rep*. 17:1184-1203.

Muller, L., S. Muller-Haegele, M. Mitsuhashi, W. Gooding, H. Okada, and T.L. Whiteside. 2015. Exosomes isolated from plasma of glioma patients enrolled in a vaccination trial reflect antitumor immune activity and might predict survival. *Oncoimmunology*. 4:e1008347.

Munson, P., Y.W. Lam, J. Dragon, M. MacPherson, and A. Shukla. 2018a. Exosomes from asbestos-exposed cells modulate gene expression in mesothelial cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*:fj201701291RR.

Munson, P., Y.W. Lam, M. MacPherson, S. Beuschel, and A. Shukla. 2018b. Mouse serum exosomal proteomic signature in response to asbestos exposure. *Journal of cellular biochemistry*.

Munson, P., and A. Shukla. 2015. Exosomes: Potential in Cancer Diagnosis and Therapy. *Medicines*. 2:310-327.

Nabavi, N., K.L. Bennewith, A. Churg, Y. Wang, C.C. Collins, and L. Mutti. 2016. Switching off malignant mesothelioma: exploiting the hypoxic microenvironment. *Genes & Cancer*. 7:340-354.

Neviani, P., and M. Fabbri. 2015. Exosomic microRNAs in the Tumor Microenvironment. *Frontiers in medicine*. 2:47.

Norbet, C., A. Joseph, S.S. Rossi, S. Bhalla, and F.R. Gutierrez. 2015. Asbestos-related lung disease: a pictorial review. *Current problems in diagnostic radiology*. 44:371-382.

Ohta, Y., V. Shridhar, G.P. Kalemkerian, R.K. Bright, Y. Watanabe, and H.I. Pass. 1999. Thrombospondin-1 expression and clinical implications in malignant pleural mesothelioma. *Cancer*. 85:2570-2576.

Pass, H.I., G.J. Brewer, R. Dick, M. Carbone, and S. Merajver. 2008. A phase II trial of tetrathiomolybdate after surgery for malignant mesothelioma: final results. *The Annals of thoracic surgery*. 86:383-389; discussion 390.

Pass, H.I., S.M. Levin, M.R. Harbut, J. Melamed, L. Chiriboga, J. Donington, M. Huflejt, M. Carbone, D. Chia, L. Goodglick, G.E. Goodman, M.D. Thornquist, G. Liu, M. de Perrot, M.-S. Tsao, and C. Goparaju. 2012a. Fibulin-3 as a Blood and Effusion Biomarker for Pleural Mesothelioma. *N Engl J Med*. 367:1417-1427.

Pass, H.I., S.M. Levin, M.R. Harbut, J. Melamed, L. Chiriboga, J. Donington, M. Huflejt, M. Carbone, D. Chia, L. Goodglick, G.E. Goodman, M.D. Thornquist, G. Liu, M. de Perrot, M.S. Tsao, and C. Goparaju. 2012b. Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. *N Engl J Med*. 367:1417-1427.

Pass, H.I., D. Lott, F. Lonardo, M. Harbut, Z. Liu, N. Tang, M. Carbone, C. Webb, and A. Wali. 2005. Asbestos exposure, pleural mesothelioma, and serum osteopontin levels. *N Engl J Med*. 353:1564-1573.

Pass, H.I., E.J. Stevens, H. Oie, M.G. Tsokos, A.D. Abati, P.A. Fetsch, D.J. Mew, H.W. Pogrebniak, and W.J. Matthews. 1995. Characteristics of nine newly derived mesothelioma cell lines. *The Annals of thoracic surgery*. 59:835-844.

Peinado, H., M. Aleckovic, S. Lavotshkin, I. Matei, B. Costa-Silva, G. Moreno-Bueno, M. Hergueta-Redondo, C. Williams, G. Garcia-Santos, C. Ghajar, A. Nitadori-Hoshino, C. Hoffman, K. Badal, B.A. Garcia, M.K. Callahan, J. Yuan, V.R. Martins, J. Skog, R.N. Kaplan, M.S. Brady, J.D. Wolchok, P.B. Chapman, Y. Kang, J. Bromberg, and D. Lyden. 2012. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nature medicine*. 18:883-891.

Peng, P., Y. Yan, and S. Keng. 2011. Exosomes in the ascites of ovarian cancer patients: origin and effects on anti-tumor immunity. *Oncology reports*. 25:749-762.

Perkins, T.N., P.M. Peeters, A. Shukla, I. Arijs, J. Dragon, E.F.M. Wouters, N.L. Reynaert, and B.T. Mossman. 2015. Indications for distinct pathogenic mechanisms of asbestos and silica through gene expression profiling of the response of lung epithelial cells. *Human Molecular Genetics*. 24:1374-1389.

Peterson, M.F., N. Otoc, J.K. Sethi, A. Gupta, and T.J. Antes. Integrated systems for exosome investigation. *Methods*.

Phuyal, S., N.P. Hessvik, T. Skotland, K. Sandvig, and A. Llorente. 2014. Regulation of exosome release by glycosphingolipids and flotillins. *The FEBS journal*. 281:2214-2227.

Piper, R.C., and D.J. Katzmann. 2007. Biogenesis and Function of Multivesicular Bodies. *Annual review of cell and developmental biology*. 23:519-547.

Pira, E., F. Donato, L. Maida, and G. Discalzi. 2018. Exposure to asbestos: past, present and future. *Journal of thoracic disease*. 10:S237-s245.

Porter, D.W., A.F. Hubbs, R.R. Mercer, N. Wu, M.G. Wolfarth, K. Sriram, S. Leonard, L. Battelli, D. Schwegler-Berry, S. Friend, M. Andrew, B.T. Chen, S. Tsuruoka, M. Endo, and V. Castranova. 2010. Mouse pulmonary dose- and time course-responses induced by exposure to multi-walled carbon nanotubes. *Toxicology*. 269:136-147.

Properzi, F., M. Logozzi, and S. Fais. 2013. Exosomes: the future of biomarkers in medicine. *Biomarkers in medicine*. 7:769-778.

Quail, D.F., and J.A. Joyce. 2013. Microenvironmental regulation of tumor progression and metastasis. *Nature medicine*. 19:1423-1437.

Quinn, L., S.P. Finn, S. Cuffe, and S.G. Gray. 2015. Non-coding RNA repertoires in malignant pleural mesothelioma. *Lung cancer (Amsterdam, Netherlands)*. 90:417-426.

Rabinowits, G., C. Gercel-Taylor, J.M. Day, D.D. Taylor, and G.H. Kloecker. 2009. Exosomal microRNA: a diagnostic marker for lung cancer. *Clinical lung cancer*. 10:42-46.

Rajagopal, C., and K.B. Harikumar. 2018. The Origin and Functions of Exosomes in Cancer. *Frontiers in oncology*. 8:66.

Rana, S., S. Yue, D. Stadel, and M. Zoller. 2012. Toward tailored exosomes: the exosomal tetraspanin web contributes to target cell selection. *The international journal of biochemistry & cell biology*. 44:1574-1584.

Rapisarda, V., R. Caltabiano, G. Musumeci, P. Castrogiovanni, M. Ferrante, C. Ledda, C. Lombardo, A.C.E. Graziano, V. Cardile, and C. Loreto. 2017. Analysis of fibulin-3 after exposure to asbestos-like fibers. *Environmental research*. 156:381-387.

Raposo, G., and W. Stoorvogel. 2013. Extracellular vesicles: exosomes, microvesicles, and friends. *The Journal of cell biology*. 200:373-383.

Rashed, M.H., P. Kanlikilicer, C. Rodriguez-Aguayo, M. Pichler, R. Bayraktar, E. Bayraktar, C. Ivan, J. Filant, A. Silva, B. Aslan, M. Denizli, R. Mitra, B. Ozpolat, G.A. Calin, A.K. Sood, M.F. Abd-Ellah, G.K. Helal, and G.L. Berestein. 2017. Exosomal miR-940 maintains SRC-mediated oncogenic activity in cancer cells: a possible role for exosomal disposal of tumor suppressor miRNAs. *Oncotarget*. 8:20145-20164.

Reale, F.R., T.W. Griffin, J.M. Compton, S. Graham, P.L. Townes, and A. Bogden. 1987. Characterization of a human malignant mesothelioma cell line (H-MESO-1): a biphasic solid and ascitic tumor model. *Cancer research*. 47:3199-3205.

Reid, G. 2015. MicroRNAs in mesothelioma: from tumour suppressors and biomarkers to therapeutic targets. *Journal of thoracic disease*. 7:1031-1040.

Reid, G., M.E. Pel, M.B. Kirschner, Y.Y. Cheng, N. Mugridge, J. Weiss, M. Williams, C. Wright, J.J. Edelman, M.P. Vallely, B.C. McCaughan, S. Klebe, H. Brahmbhatt, J.A. MacDiarmid, and N. van Zandwijk. 2013. Restoring expression of miR-16: a novel approach to therapy for malignant pleural mesothelioma. *Annals of oncology : official journal of the European Society for Medical Oncology*. 24:3128-3135.

Ridder, K., A. Sevko, J. Heide, M. Dams, A.K. Rupp, J. Macas, J. Starmann, M. Tjwa, K.H. Plate, H. Sultmann, P. Altevogt, V. Umansky, and S. Momma. 2015. Extracellular vesicle-mediated transfer of functional RNA in the tumor microenvironment. *Oncoimmunology*. 4:e1008371.

Robinson, B.W., and R.A. Lake. 2005. Advances in malignant mesothelioma. *N Engl J Med*. 353:1591-1603.

Roe, O.D., E. Anderssen, H. Sandeck, T. Christensen, E. Larsson, and S. Lundgren. 2010. Malignant pleural mesothelioma: genome-wide expression patterns reflecting general resistance mechanisms and a proposal of novel targets. *Lung cancer (Amsterdam, Netherlands)*. 67:57-68.

Roeser, H.P., G.R. Lee, S. Nacht, and G.E. Cartwright. 1970. The role of ceruloplasmin in iron metabolism. *Journal of Clinical Investigation*. 49:2408-2417.

Rood, I.M., J.K. Deegens, M.L. Merchant, W.P. Tamboer, D.W. Wilkey, J.F. Wetzels, and J.B. Klein. 2010. Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome. *Kidney international*. 78:810-816.

Rosenberg, S.A., and N.P. Restifo. 2015. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science (New York, N.Y.)*. 348:62-68.

Rossini, M., P. Rizzo, I. Bononi, A. Clementz, R. Ferrari, F. Martini, and M.G. Tognon. 2018. New Perspectives on Diagnosis and Therapy of Malignant Pleural Mesothelioma. *Frontiers in oncology*. 8:91.

Roushdy-Hammady, I., J. Siegel, S. Emri, J.R. Testa, and M. Carbone. 2001. Genetic-susceptibility factor and malignant mesothelioma in the Cappadocian region of Turkey. *Lancet*. 357:444-445.

Ryan, A.J., J.L. Larson-Casey, C. He, S. Murthy, and A.B. Carter. 2014. Asbestos-induced disruption of calcium homeostasis induces endoplasmic reticulum stress in macrophages. *J Biol Chem*. 289:33391-33403.

Saenz-Cuesta, M., I. Osorio-Querejeta, and D. Otaegui. 2014. Extracellular Vesicles in Multiple Sclerosis: What are They Telling Us? *Frontiers in cellular neuroscience*. 8:100.

Safaei, R., B.J. Larson, T.C. Cheng, M.A. Gibson, S. Otani, W. Naerdemann, and S.B. Howell. 2005. Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. *Molecular cancer therapeutics*. 4:1595-1604.

Sahoo, S., and D.W. Losordo. 2014. Exosomes and cardiac repair after myocardial infarction. *Circulation research*. 114:333-344.

Sampaio, N.G., L. Cheng, and E.M. Eriksson. 2017. The role of extracellular vesicles in malaria biology and pathogenesis. *Malaria journal*. 16:245.

Sarafan-Vasseur, N., A. Lamy, J. Bourguignon, F. Le Pessot, P. Hieter, R. Sesboue, C. Bastard, T. Frebourg, and J.M. Flaman. 2002. Overexpression of B-type cyclins alters chromosomal segregation. *Oncogene*. 21:2051-2057.

Schramm, A., I. Opitz, S. Thies, B. Seifert, H. Moch, W. Weder, and A. Soltermann. 2010. Prognostic significance of epithelial-mesenchymal transition in malignant pleural mesothelioma. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery*. 37:566-572.

Sekido, Y. 2013. Molecular pathogenesis of malignant mesothelioma. *Carcinogenesis*. 34:1413-1419.

Selmaj, I., M.P. Mycko, C.S. Raine, and K.W. Selmaj. 2017. The role of exosomes in CNS inflammation and their involvement in multiple sclerosis. *Journal of neuroimmunology*. 306:1-10.

Sen, D. 2015. Working with asbestos and the possible health risks. *Occupational medicine (Oxford, England)*. 65:6-14.

Sezgi, C., M. Taylan, H. Selimoglu Sen, O. Evliyaoğlu, H. Kaya, O. Abakay, A. Abakay, A.C. Tanrıkulu, and A. Senyiğit. 2014. Oxidative Status and Acute Phase Reactants in Patients with Environmental Asbestos Exposure and Mesothelioma. *The Scientific World Journal*. 2014:902748.

Shahabipour, F., N. Barati, T.P. Johnston, G. Derosa, P. Maffioli, and A. Sahebkar. 2017. Exosomes: Nanoparticulate tools for RNA interference and drug delivery. *Journal of cellular physiology*. 232:1660-1668.

Shannahan, J.H., O. Alzate, W.M. Winnik, D. Andrews, M.C. Schladweiler, A.J. Ghio, S.H. Gavett, and U.P. Kodavanti. 2012. Acute phase response, inflammation and metabolic syndrome biomarkers of Libby asbestos exposure. *Toxicology and applied pharmacology*. 260:105-114.

Shukla, A., M.W. Bosenberg, M.B. MacPherson, K.J. Butnor, N.H. Heintz, H.I. Pass, M. Carbone, J.R. Testa, and B.T. Mossman. 2009a. Activated cAMP Response Element Binding Protein Is Overexpressed in Human Mesotheliomas and Inhibits Apoptosis. *The American journal of pathology*. 175:2197-2206.

Shukla, A., J.M. Hillegass, M.B. MacPherson, S.L. Beuschel, P.M. Vacek, K.J. Butnor, H.I. Pass, M. Carbone, J.R. Testa, N.H. Heintz, and B.T. Mossman. 2011. ERK2 is essential for the

growth of human epithelioid malignant mesotheliomas. *International journal of cancer*. 129:1075-1086.

Shukla, A., M.B. MacPherson, J. Hillegass, M.E. Ramos-Nino, V. Alexeeva, P.M. Vacek, J.P. Bond, H.I. Pass, C. Steele, and B.T. Mossman. 2009b. Alterations in gene expression in human mesothelial cells correlate with mineral pathogenicity. *American journal of respiratory cell and molecular biology*. 41:114-123.

Shukla, A., J.M. Miller, C. Cason, M. Sayan, M.B. MacPherson, S.L. Beuschel, J. Hillegass, P.M. Vacek, H.I. Pass, and B.T. Mossman. 2013. Extracellular signal-regulated kinase 5: a potential therapeutic target for malignant mesotheliomas. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 19:2071-2083.

Sioud, M. 2015. RNA interference: mechanisms, technical challenges, and therapeutic opportunities. *Methods in molecular biology (Clifton, N.J.)*. 1218:1-15.

Sivadasan, P., M.K. Gupta, G.J. Sathe, L. Balakrishnan, P. Palit, H. Gowda, A. Suresh, M.A. Kuriakose, and R. Sirdeshmukh. 2015. Human salivary proteome - a resource of potential biomarkers for oral cancer. *Journal of proteomics*.

Skotland, T., K. Sandvig, and A. Llorente. 2017. Lipids in exosomes: Current knowledge and the way forward. *Progress in lipid research*. 66:30-41.

Smalheiser, N.R. 2007. Exosomal transfer of proteins and RNAs at synapses in the nervous system. *Biology direct*. 2:35.

Spitsyn, V.A., G.V. Tsurikova, and I.S. Afanas'eva. 1992. [Gene manifestations in negative man-made environment: a selective genetically determined sensitivity to asbestos]. *Vestnik Rossiiskoi akademii meditsinskikh nauk*:46-52.

Sterzenbach, U., U. Putz, L.H. Low, J. Silke, S.S. Tan, and J. Howitt. 2017. Engineered Exosomes as Vehicles for Biologically Active Proteins. *Molecular therapy : the journal of the American Society of Gene Therapy*. 25:1269-1278.

Sun, Y., S. Liu, Z. Qiao, Z. Shang, Z. Xia, X. Niu, L. Qian, Y. Zhang, L. Fan, C.X. Cao, and H. Xiao. 2017. Systematic comparison of exosomal proteomes from human saliva and serum for the detection of lung cancer. *Analytica chimica acta*. 982:84-95.

Sung, B.H., T. Ketova, D. Hoshino, and A. Zijlstra. 2015. Directional cell movement through tissues is controlled by exosome secretion. 6:7164.

Syn, N.L., L. Wang, E.K. Chow, C.T. Lim, and B.C. Goh. 2017. Exosomes in Cancer Nanomedicine and Immunotherapy: Prospects and Challenges. *Trends in biotechnology*. 35:665-676.

Szatanek, R., M. Baj-Krzyworzeka, J. Zimoch, M. Lekka, M. Siedlar, and J. Baran. 2017. The Methods of Choice for Extracellular Vesicles (EVs) Characterization. *International journal of molecular sciences*. 18.

Szczepanski, M.J., M. Szajnik, A. Welsh, T.L. Whiteside, and M. Boyiadzis. 2011. Blast-derived microvesicles in sera from patients with acute myeloid leukemia suppress natural killer cell function via membrane-associated transforming growth factor-beta1. *Haematologica*. 96:1302-1309.

Takahashi, R.U., M. Prieto-Vila, A. Hironaka, and T. Ochiya. 2017. The role of extracellular vesicle microRNAs in cancer biology. *Clinical chemistry and laboratory medicine*. 55:648-656.

Tamminen, J.A., M. Myllarniemi, M. Hyytiainen, J. Keski-Oja, and K. Koli. 2012. Asbestos exposure induces alveolar epithelial cell plasticity through MAPK/Erk signaling. *Journal of cellular biochemistry*. 113:2234-2247.

Tang, M.K., and A.S. Wong. 2015. Exosomes: Emerging biomarkers and targets for ovarian cancer. *Cancer letters*.

Teeguarden, J.G., B.J. Webb-Robertson, K.M. Waters, A.R. Murray, E.R. Kisin, S.M. Varnum, J.M. Jacobs, J.G. Pounds, R.C. Zanger, and A.A. Shvedova. 2011. Comparative proteomics and pulmonary toxicity of instilled single-walled carbon nanotubes, crocidolite asbestos, and ultrafine carbon black in mice. *Toxicological sciences : an official journal of the Society of Toxicology*. 120:123-135.

Teixeira, J.H., A.M. Silva, M.I. Almeida, M.A. Barbosa, and S.G. Santos. 2016. Circulating extracellular vesicles: Their role in tissue repair and regeneration. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis*.

Thayanithy, V., V. Babatunde, E.L. Dickson, P. Wong, S. Oh, X. Ke, A. Barlas, S. Fujisawa, Y. Romin, A.L. Moreira, R.J. Downey, C.J. Steer, S. Subramanian, K. Manova-Todorova, M.A. Moore, and E. Lou. 2014. Tumor exosomes induce tunneling nanotubes in lipid raft-enriched regions of human mesothelioma cells. *Experimental cell research*. 323:178-188.

Thompson, J.K., M.B. MacPherson, S.L. Beuschel, and A. Shukla. Asbestos-Induced Mesothelial to Fibroblastic Transition Is Modulated by the Inflammasome. *The American journal of pathology*. 187:665-678.

Thompson, J.K., M.B. MacPherson, S.L. Beuschel, and A. Shukla. 2017. Asbestos-Induced Mesothelial to Fibroblastic Transition Is Modulated by the Inflammasome. *The American journal of pathology*. 187:665-678.

Thompson, J.K., A. Shukla, A.L. Leggett, P.B. Munson, J.M. Miller, M.B. MacPherson, S.L. Beuschel, H.I. Pass, and A. Shukla. 2018. Extracellular signal regulated kinase 5 and inflammasome in progression of mesothelioma. *Oncotarget*. 9:293-305.

Thompson, J.K., C.M. Westbom, M.B. MacPherson, B.T. Mossman, N.H. Heintz, P. Spiess, and A. Shukla. 2014. Asbestos modulates thioredoxin-thioredoxin interacting protein interaction to regulate inflammasome activation. *Part Fibre Toxicol*. 11:24.

Tian, Y., S. Li, J. Song, T. Ji, M. Zhu, G.J. Anderson, J. Wei, and G. Nie. 2014. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials*. 35:2383-2390.

Timpl, R., T. Sasaki, G. Kostka, and M.-L. Chu. 2003. Fibulins: a versatile family of extracellular matrix proteins. *Nat Rev Mol Cell Biol*. 4:479-489.

Trams, E.G., C.J. Lauter, N. Salem, Jr., and U. Heine. 1981. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochimica et biophysica acta*. 645:63-70.

Tranchant, R., L. Quetel, A. Tallet, C. Meiller, A. Renier, L. de Koning, A. de Reynies, F. Le Pimpec-Barthes, J. Zucman-Rossi, M.C. Jaurand, and D. Jean. 2017. Co-occurring Mutations of Tumor Suppressor Genes, LATS2 and NF2, in Malignant Pleural Mesothelioma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 23:3191-3202.

Truini, A., S. Coco, A. Alama, C. Genova, C. Sini, M.G. Dal Bello, G. Barletta, E. Rijavec, G. Burrafato, F. Boccardo, and F. Grossi. 2014. Role of microRNAs in malignant mesothelioma. *Cellular and Molecular Life Sciences*. 71:2865-2878.

Vader, P., X.O. Breakefield, and M.J. Wood. 2014. Extracellular vesicles: emerging targets for cancer therapy. *Trends in molecular medicine*. 20:385-393.

Valenti, R., V. Huber, M. Iero, P. Filipazzi, G. Parmiani, and L. Rivoltini. 2007. Tumor-released microvesicles as vehicles of immunosuppression. *Cancer research*. 67:2912-2915.

Valenzuela, M.M., H.R. Ferguson Bennit, A. Gonda, C.J. Diaz Osterman, A. Hibma, S. Khan, and N.R. Wall. 2015. Exosomes Secreted from Human Cancer Cell Lines Contain Inhibitors of

Apoptosis (IAP). *Cancer microenvironment : official journal of the International Cancer Microenvironment Society*.

van der Pol, E., A.N. Boing, P. Harrison, A. Sturk, and R. Nieuwland. 2012. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacological reviews*. 64:676-705.

van Zandwijk, N., N. Pavlakis, S.C. Kao, A. Linton, M.J. Boyer, S. Clarke, Y. Huynh, A. Chrzanowska, M.J. Fulham, D.L. Bailey, W.A. Cooper, L. Kritharides, L. Ridley, S.T. Pattison, J. MacDiarmid, H. Brahmbhatt, and G. Reid. 2017. Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. *The Lancet. Oncology*. 18:1386-1396.

Vella, L.J. 2014. The emerging role of exosomes in epithelial-mesenchymal-transition in cancer. *Frontiers in oncology*. 4:361.

Vicencio, J.M., D.M. Yellon, V. Sivaraman, D. Das, C. Boi-Doku, S. Arjun, Y. Zheng, J.A. Riquelme, J. Kearney, V. Sharma, G. Multhoff, A.R. Hall, and S.M. Davidson. 2015. Plasma exosomes protect the myocardium from ischemia-reperfusion injury. *Journal of the American College of Cardiology*. 65:1525-1536.

Viteri, S., and R. Rosell. 2017. An innovative mesothelioma treatment based on miR-16 mimic loaded EGFR targeted minicells (TargomiRs). *Translational Lung Cancer Research*:S1-S4.

Vlassov, A.V., S. Magdaleno, R. Setterquist, and R. Conrad. 2012. Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochimica et Biophysica Acta (BBA) - General Subjects*. 1820:940-948.

Vogelzang, N.J., J.J. Rusthoven, J. Symanowski, C. Denham, E. Kaukel, P. Ruffie, U. Gatzemeier, M. Boyer, S. Emri, C. Manegold, C. Niyikiza, and P. Paoletti. 2003. Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 21:2636-2644.

Vyas, N., and J. Dhawan. 2017. Exosomes: mobile platforms for targeted and synergistic signaling across cell boundaries. *Cellular and molecular life sciences : CMLS*. 74:1567-1576.

Wagner, J.C., C.A. Sleggs, and P. Marchand. 1960. Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. *British journal of industrial medicine*. 17:260-271.

Wang, D.W., Y.Q. Wang, and H.S. Shu. 2018. MiR-16 inhibits pituitary adenoma cell proliferation via the suppression of ERK/MAPK signal pathway. *European review for medical and pharmacological sciences*. 22:1241-1248.

Webber, J., R. Steadman, M.D. Mason, Z. Tabi, and A. Clayton. 2010. Cancer exosomes trigger fibroblast to myofibroblast differentiation. *Cancer research*. 70:9621-9630.

Welker, L., M. Muller, O. Holz, E. Vollmer, H. Magnussen, and R.A. Jorres. 2007. Cytological diagnosis of malignant mesothelioma--improvement by additional analysis of hyaluronic acid in pleural effusions. *Virchows Archiv : an international journal of pathology*. 450:455-461.

Whiteside, T.L. 2017. Exosomes carrying immunoinhibitory proteins and their role in cancer. *Clinical and experimental immunology*.

Whiteside, T.L. 2018. The emerging role of plasma exosomes in diagnosis, prognosis and therapies of patients with cancer. *Contemporary oncology (Poznan, Poland)*. 22:38-40.

Wolf, P. 1967. The Nature and Significance of Platelet Products in Human Plasma. *British Journal of Haematology*. 13:269-288.

Wolfers, J., A. Lozier, G. Raposo, A. Regnault, C. Thery, C. Masurier, C. Flament, S. Pouzieux, F. Faure, T. Tursz, E. Angevin, S. Amigorena, and L. Zitvogel. 2001. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nature medicine*. 7:297-303.

Wu, X., T. Zheng, and B. Zhang. 2017. Exosomes in Parkinson's Disease. *Neuroscience bulletin*. 33:331-338.

Wu, Y., W. Deng, and D.J. Klinke. 2015. Exosomes: Improved methods to characterize their morphology, RNA content, and surface protein biomarkers. *The Analyst*. 140:6631-6642.

Wubbolts, R., R.S. Leckie, P.T. Veenhuizen, G. Schwarzmann, W. Mobius, J. Hoernschemeyer, J.W. Slot, H.J. Geuze, and W. Stoorvogel. 2003. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J Biol Chem*. 278:10963-10972.

Xu, F., X. Zhang, Y. Lei, X. Liu, Z. Liu, T. Tong, and W. Wang. 2010. Loss of Repression of HuR Translation by miR-16 May Be Responsible for the Elevation of HuR in Human Breast Carcinoma. *Journal of cellular biochemistry*. 111:727-734.

Yan, S., B. Han, S. Gao, X. Wang, Z. Wang, F. Wang, J. Zhang, D. Xu, and B. Sun. 2017. Exosome-encapsulated microRNAs as circulating biomarkers for colorectal cancer. *Oncotarget*.

Yang, F., X. Liao, Y. Tian, and G. Li. 2017. Exosome separation using microfluidic systems: size-based, immunoaffinity-based and dynamic methodologies. *Biotechnology journal*. 12.

Yang, T., P. Martin, B. Fogarty, A. Brown, K. Schurman, R. Phipps, V.P. Yin, P. Lockman, and S. Bai. 2015. Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in danio rerio. *Pharmaceutical research*. 32:2003-2014.

Yap, T.A., J.G. Aerts, S. Popat, and D.A. Fennell. 2017. Novel insights into mesothelioma biology and implications for therapy. *Nature reviews. Cancer*. 17:475-488.

Yeo, R.W., R.C. Lai, B. Zhang, S.S. Tan, Y. Yin, B.J. Teh, and S.K. Lim. 2013. Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. *Adv Drug Deliv Rev*. 65:336-341.

Yin, W., S. Ouyang, Y. Li, B. Xiao, and H. Yang. 2013. Immature dendritic cell-derived exosomes: a promise subcellular vaccine for autoimmunity. *Inflammation*. 36:232-240.

Young, L.E., A.E. Moore, L. Sokol, N. Meisner-Kober, and D.A. Dixon. 2012. The mRNA stability factor HuR inhibits microRNA-16 targeting of COX-2. *Molecular cancer research : MCR*. 10:167-180.

Yu, D.D., Y. Wu, H.Y. Shen, M.M. Lv, W.X. Chen, X.H. Zhang, S.L. Zhong, J.H. Tang, and J.H. Zhao. 2015. Exosomes in Development, Metastasis and Drug Resistance of Breast Cancer. *Cancer science*.

Zarovni, N., A. Corrado, P. Guazzi, D. Zocco, E. Lari, G. Radano, J. Muhhina, C. Fondelli, J. Gavrilova, and A. Chiesi. 2015. Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches. *Methods*.

Zhang, H.G., and W.E. Grizzle. 2011. Exosomes and cancer: a newly described pathway of immune suppression. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 17:959-964.

Zhao, L., W. Liu, J. Xiao, and B. Cao. 2015. The role of exosomes and "exosomal shuttle microRNA" in tumorigenesis and drug resistance. *Cancer letters*. 356:339-346.

Zhou, J., G. Gong, H. Tan, F. Dai, X. Zhu, Y. Chen, J. Wang, Y. Liu, P. Chen, X. Wu, and J. Wen. 2015a. Urinary microRNA-30a-5p is a potential biomarker for ovarian serous adenocarcinoma. *Oncology reports*. 33:2915-2923.

Zhou, X., W. Zhu, H. Li, W. Wen, W. Cheng, F. Wang, Y. Wu, L. Qi, Y. Fan, Y. Chen, Y. Ding, J. Xu, J. Qian, Z. Huang, T. Wang, D. Zhu, Y. Shu, and P. Liu. 2015b. Diagnostic value of a plasma microRNA signature in gastric cancer: a microRNA expression analysis. *Sci Rep*. 5:11251.

Zomer, A., C. Maynard, F.J. Verweij, A. Kamermans, R. Schafer, E. Beerling, R.M. Schiffelers, E. de Wit, J. Berenguer, S.I. Ellenbroek, T. Wurdinger, D.M. Pegtel, and J. van Rheenen. 2015. In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell*. 161:1046-1057.

Zukowski, R., and R. Gaze. 1959. Tensile Strength of Asbestos. *Nature*. 183:35-37.