Identification And Molecular Analysis Of DNA In Exosomes

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EXOSOMES

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Dedication

First and foremost, I would like to dedicate this dissertation to my parents, Leslie and John. My parents have always provided me with unwavering confidence and support, which has enabled me to achieve every goal I've set for myself thus far. While I cannot put into words how much I appreciate all they have sacrificed for me, I can at least offer my immeasurable gratitude. Thank you for helping me get to where I am today, I could not have done any of it without you.

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IDENTIFICATION AND MOLECULAR ANALYSIS OF DNA IN EXOSOMES

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Exosomes are heterogeneous nanoparticles 50-150nm in diameter. Exosomes contain many functional cargo components, such as protein, DNA, and RNA. While protein and RNA exosome content has been extensively studied, very little work has been done to characterize exosomal DNA. Here, we demonstrate that exosomal DNA is heterogeneous and its packaging into exosomes is dependent on the cell of origin. Furthermore, through a rigorous assessment of various isolation methods, we identify Size Exclusion Chromatography (SEC) as the best method for the isolation of exosomal DNA for downstream applications. Additionally, we evaluate the methylation status of exosomal DNA and demonstrate that exosomal DNA is both methylated and fully recapitulates the methylation patterns observed in the cells of origin. We also propose a potential mechanism for DNA packaging into exosomes by disruption of the nuclear membrane. Finally, we investigated the ability of exosomes to induce paracrine DNA damage responses (DDR) in treatment-naïve cells. We explore the specificity of exosome-induced DDR to exosomes released by damaged cancer cells, and provide a potential molecular mechanism of action via the shuttling of activated DDR pathway proteins.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Biogenesis, Characteristics, and Functions of Exosomes

Early Exosome Discovery and Overview

The initial identification of the class of nanoparticles now known as exosomes occurred almost 40 years ago. While there were earlier accounts of small vesicles secreted from certain cell types, such as chondrocytes[1,2] and platelets[3], the term 'exosome' was not used until Trams et al. published the first account of membrane particles isolated from biological fluid in 1981[4]. Their landmark paper was the first to state that exosomes could come from a wide variety of cell types, including both normal and neoplastic, as part of a general biological process that could preferentially sort cargo for packaging and transfer it to recipient cells[4]. Thus, this was the first suggestion of the potential of exosomes for intercellular signaling and, further, for their use as therapeutic delivery vehicles.

Two years later, in 1983, two seminal papers were published that served as the true foundation for the field of exosome research. Both investigated transferrin receptors in reticulocytes, and showed that the receptors were released from cells via vesicles[5,6]. Additionally the first of those papers, published by Harding and Stahl, showed for the first time that multivesicular endosomes, or MVEs, contained small vesicles that were then ejected into the extracellular environment upon fusion with the plasma membrane, becoming the first study to demonstrate the general mechanism of exosome release[5]. At the time, late endosomes were only thought to be pre-degradative compartments, with their contents designated specifically for lysosomal degradation. Therefore, the discovery that certain late endosomes could fuse with the cellular membrane and release contents was quite controversial, since most believed

that any vesicles retrieved by ultracentrifugation were membrane components of dying cells in culture. More recently, electron microscopy has been used to demonstrate membrane fusion events between late endosomes and the plasma membranes of living cells[7,8], validating the initial findings. The name exosome was officially linked specifically to 40-100nm nanoparticles released via MVE fusion in Rose Johnstone's 1987 paper, which further characterized the release pathway as being distinct from cellular stress responses[9].

In the decades since exosomes were first identified the field has rapidly expanded, generating a massive amount of data regarding the characterization, functions, and applications of exosomes. Exosomes are now known to be released from virtually every cell type, including but not limited to immune cells[7,8], neurons[10,11], stem cells[12,13], and cancer cells[14–16]. Exosomes have also been found to be released by prokaryotes and eukaryotes in addition to mammalian cells[17,18]. Importantly, especially for clinical applications, exosomes have been isolated from a wide variety of biological fluids, such as serum[19,20], urine[21], breast milk[22], amniotic fluid[23], saliva[24], semen[25], and cerebrospinal fluid[26]. The therapeutic applications of these bodily fluid-derived exosomes are still being explored, but the potential for diagnostic and therapeutic implementations of exosomes for many different diseases is enormous.

Beginning with the groundbreaking study published by Raposo et al. in 1996, in which it was shown that exosomes could induce adaptive immune responses by acting as antigen presenting vesicles via MHC class II complexes present on their membranes[8], exosomes have been demonstrated to have a wide variety of functions. In addition to their roles in immunity, exosomes have also been implicated in cellular

signaling and biological processes, under both normal physiological and pathological conditions. It is these functional characteristics of exosomes that drive much of the research in the field today, on a breadth of topics including stem cell maintenance[12], tissue repair[27], neuronal synaptic responses[28], or most relevant for this work, tumorigenesis[29–32], among others. In addition to cancer, exosomes have been linked to a variety of other diseases, including HIV[33], Alzheimer's[34], and Parkinson's disease[35], and are under investigation for both diagnostic and therapeutic applications.

As exosome research has become more widespread, many advances have been made regarding their isolation and identification. There are now many different exosome isolation methods, which differ in efficacy depending on the desired downstream application. These methods will be discussed in greater detail subsequently. In parallel with the development of improved exosome isolation techniques, exosome-specific markers have also been identified since exosomes can now be obtained with greater purity. The most commonly used exosome markers are known as tetraspanins, transmembrane proteins with a variety of functions including cell adhesion, motility, and membrane fusion[36]. The first tetraspanin to be identified as specifically associated with exosomes was CD9, which was discovered by Théry et al. in the exosome pellets of dendritic cells in 1999[7]. Since then, CD63 and CD81 have most often been cited as the most classical markers of exosomes[37,38], however there has been evidence of other classes of microvesicles containing some of the same tetraspanins, likely due to the wide distribution of tetraspanin expression throughout the plasma membrane[39]. Therefore, tetraspanins are necessary but no

longer sufficient to precisely identify exosomes, and other markers have been identified to supplement tetraspanin expression, such as flotillin, TSG101, integrins, lactadherin, and RAB5/RAB7[40,41]. Additionally, exosomes are characterized by the absence of certain markers, such as calnexin, GM130, and cytochrome C, to indicate they are not apoptotic bodies[42].

Finally, exosomes have been shown to have heterogenous and diverse cargo components that generally reflect the cell of origin and have a variety of functions and potential uses. While these will later be described in more detail, what follows is a brief description of some of the major categories of exosome contents. Perhaps the most commonly studied class of exosome cargo is protein. Many proteins have been identified either on the membranes of exosomes, such as the aforementioned tetraspanins[36], or encapsulated within them[40,43]. Many species of RNAs have also been demonstrated to be carried by exosomes, including mRNA, miRNA, tRNA, and rRNA, YRNA, and Vault RNA[30,43–45]. Another important nucleic acid, DNA, is also present in exosomes and was first identified (in double-stranded form) as an exosomal cargo component by the Kalluri laboratory in 2014[46]. That finding was swiftly corroborated by other groups, and ssDNA and mtDNA have also been shown in exosomes[11,47–49]. These studies set the early foundation for much of the work done in this project. Other exosomal cargo components include lipids, cytoskeleton components, and enzymes[40,43,44,50]. A growing area of interest of therapeutic relevance is the loading of exosomes with specific cargo for delivery to target cells. One example of this was work done in our lab that utilized exosomes to deliver siRNA targeting oncogenic KRASG12D to pancreatic ductal adenocarcinoma (PDAC) tumors

in mice, resulting in extensive tumor burden reduction and suppression of KRASG12D expression[51]. This work is now in clinical trial, and many other studies involving exosomes as therapy delivery vehicles are ongoing.
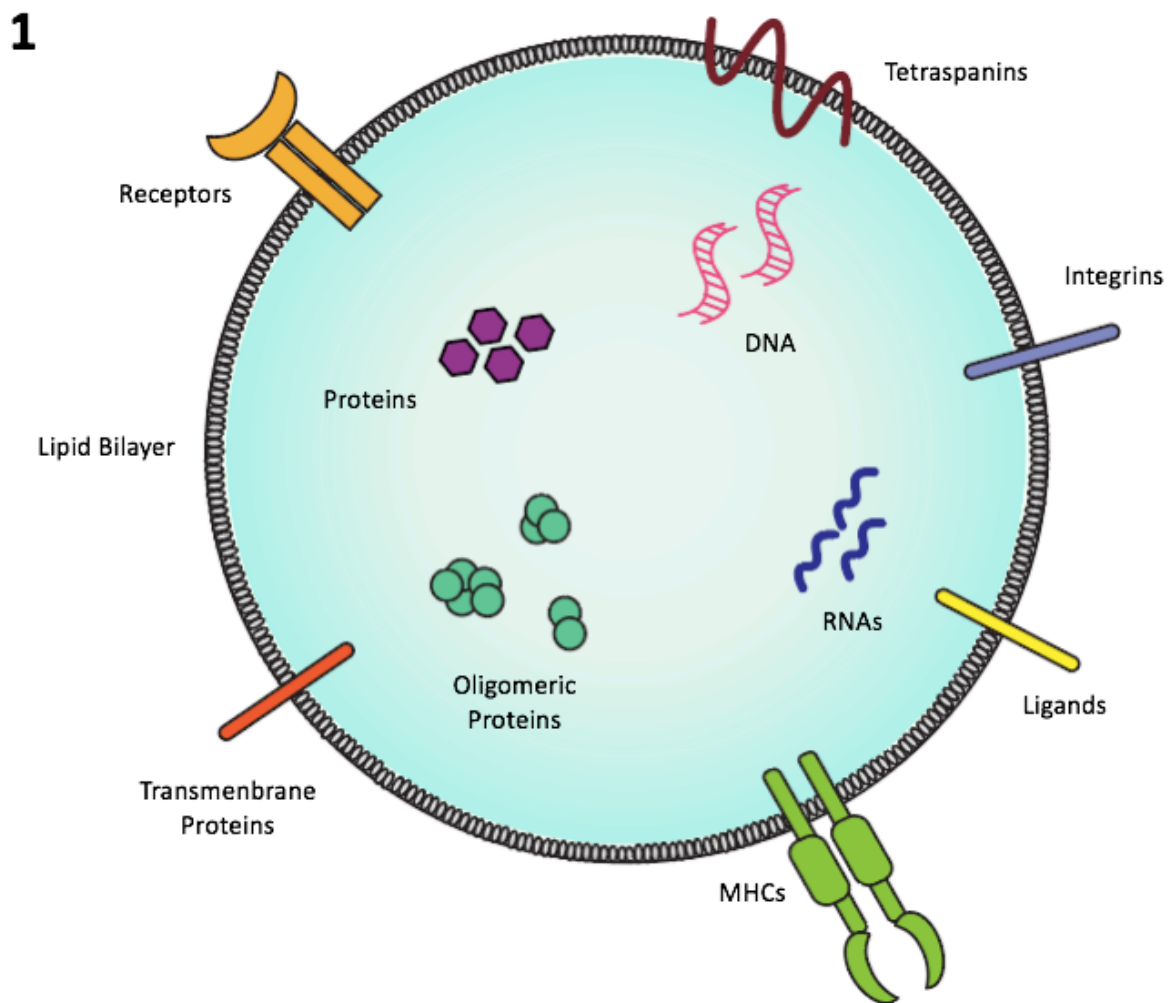
Figure 1. Exosome Components.

Exosomes contain heterogenous cargo components, which can include RNA, DNA and both intraluminal and membrane proteins.

Mechanism of Exosome Biogenesis

Since Harding and Stahl first observed exosome release via MVE fusion with the cellular membrane[5], much has been discovered regarding the biological processes of exosome biogenesis and release. Generally speaking, the pathway involves multivesicular bodies containing intraluminal vesicles, or exosomes, forming and subsequently fusing with the plasma membrane of the cell of origin, releasing the exosomes into the intercellular space[52]. Exosome biogenesis is comprised of three major stages, the formation of endocytic vesicles, multi-vesicular body (MVB) generation via the inward budding of the endosomal membrane to form intraluminal vesicles (ILVs), and the fusion of MVBs with the parent cell's plasma membrane with resultant exosome release[50]. While many of the proteins and molecules involved in the biogenesis and release of exosomes have been identified, due to technical limitations there is still a lack of clarity regarding the roles of certain molecules at each step. There is also conflicting information regarding which molecules are important for exosome biogenesis, possibly due to factors such as choice of isolation method or cell type-specific dependencies. Additionally, since much of the exosome biogenesis pathway has only recently been defined, there are likely still components that have not yet been described.

Exosome formation begins with the maturation of early endosomes into late endosomes. During the maturation process, the endosomal membrane invaginates and forms ILVs within the lumen of the endosome, forming an MVB[53]. The ESCRT complex machinery, a collection of about 30 proteins assembled into 4 complexes[50],

is integral to this process. The ESCRT-0 complex recognizes and traps ubiquitinated transmembrane proteins in the endosomal membrane, while the ESCRT I & II complexes are involved in membrane deformation and the formation of buds with sorted cargo[54]. The ESCRT III complex is responsible for separating the newly formed vesicles from the membrane[54]. Several distinct ESCRT proteins have been clearly demonstrated to be important for the successful secretion of exosomes, namely Hrs, TSG101, and STAM1[55]. Additionally, the related protein ALIX has been implicated in the loading of cargo and selection of MVBs for maturation and secretion[55].

The ESCRT proteins (ESCRTs) may not be completely necessary for exosome release, as silencing key subunits of all 4 ESCRT complexes does not entirely eliminate the formation of ILVs[56]. Tetraspanins, specifically CD9, CD82, Tspan8, and CD63, have been implicated in ESCRT-independent exosome release[57–59], as has the lysosome/late endosome associated protein SIMPLE[60]. Lipids have also been shown to work together with these proteins as important actors in vesicular formation and transport processes such as membrane curvature, invagination, fission and fusion[61]. The role of lipids in exosome formation is supported by studies targeting lipid modifying enzymes, such as the inhibition of sphingomyelinase 2 (nSMase2) which has been shown to reduce exosome release, potentially due to the subsequent lack of formation of ceramide microdomains that cause membrane budding to occur[62]. Other lipid modifying enzymes, such as phospholipase D2 (PLD2) and diacylglycerol kinase α (DGKα), have also been identified as having a role in regulating ILV formation in exosome biogenesis[63,64]. The maturation of endosomes into MVBs is likely to rely

on these different components either separately or combined based on the conditions present in a given cell type and microenvironment[65].

Once an endosome has matured into an MVB, it will either be targeted to lysosomes, which results in the degradation of its contents, or transported to the plasma membrane of the cell for fusion and release. How MVBs are designated for either degradation or secretion remains a mystery, but some groups have identified a few potential markers, such as post-translational modifications[66]. If an MVB avoids degradation, it must physically move through the cytosol to the plasma membrane. This process is mediated by MVB interactions with cytoskeletal components of the cell, namely actin and microtubules[67,68]. Additionally, the Rab GTPase family is known to be involved in several steps of the membrane trafficking process, including budding, transport along actin and tubulin, and membrane fusion. Several of these Rab GTPases have been implicated specifically in the exosome biogenesis pathway, such as Rab11, Rab35, and Rab27a/b, which have been the most characterized[69,70]. Other small GTPases may also be involved in exosome release, such as the RhoGTPase citron kinase[71]. It is worth mentioning that while all of these molecules have been implicated in exosome release, some studies have shown that their involvement may be dependent on cell type[72–74], meaning there is likely at least some degree of functional redundancy in the MVB trafficking pathway. Additionally, the high likelihood that there are as-yet unidentified additional molecules involved in the process demonstrates the need for further clarity regarding the MVB-to-membrane transport mechanisms.

Once the MVBs arrive at the plasma membrane, several steps must occur to achieve proper membrane fusion and exosome release. Multiple proteins have been identified as fusion facilitators, in addition to providing docking specificity. Chief among these are the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), as well as tethering factors, Rabs, and select other Ras GTPases[75]. SNAREs have been shown to enable vesicles to fuse with specific target membranes, either the plasma membrane of the cell or the membrane of another organelle[76]. The SNARE family is split into two classes, R-SNAREs and Q-SNAREs, and fusion usually requires one R-SNARE and three Q-SNAREs to form a complex which then enables the MVB to dock at the plasma membrane[75]. SNAREs act together with Rab GTPases and tethering factors to complete vesicle targeting and fusion with high efficiency[77]. It is thought that Rab GTPases present on the surface of vesicles recruit the cytosolic tethers to their membranes, which work in concert with tethers and SNARE complexes on the target membrane to achieve fusion[75]. The precise mechanisms of this process remain an active field of study.

Exosome release has been shown to be at least somewhat dependent on the cellular microenvironment and relies on several aspects of cellular homeostasis. It has been reported that cells under stress release more exosomes in a variety of contexts, though the exact mechanisms are not always clear. For example, dying or senescent cells were shown to secrete exosomes at a higher rate when compared with healthy cells[78,79]. Additionally, cells subjected to DNA damaging agents and hypoxia were also observed to release more exosomes[80,81]. While the exact purpose(s) of increased exosome release in the context of stress remain unclear, it has been proposed that

this could be a mechanism of releasing waste products detrimental to the cells or as a means of sending a stress signal to neighboring cells as a form of intercellular communication[82].

Exosome Cargo

The functions of exosomes are all mediated by the biological components contained either within or on exosomes. Exosomes have been shown to contain heterogeneous and diverse cargo, which is largely dependent on the cell type of origin as well as the microenvironmental conditions at the time of release. The mechanisms of exosome packaging and cargo sorting remain unclear, but some progress has been made in identifying mediators that determine what is loaded into exosomes during biogenesis[83,84]. More substantial work has been done on identifying and characterizing the molecules within exosomes, which include lipids, proteins, and nucleic acids such as RNA and DNA[40].

Lipids have not only been identified as exosome cargo, but also form the exosomal membrane. The outer membrane of exosomes consists of a lipid bilayer, similar to the plasma membrane of cells[44]. Compared with cells, exosome membranes have been shown to be enriched in cholesterol, sphingomyelin, gangliosides, and desaturated lipids, while they have less phosphatidylcholine and diacylglycerol[85,86]. Exosomes have demonstrated an increased amount of phosphatidylserine on the outer leaflet of their membranes, which has been proposed to aid their uptake by recipient cells[87]. The increased rigidity of exosome membranes in comparison with cell membranes, due to their high levels of sphingomyelin, cholesterol, and desaturated lipids, has been correlated with their resistance to degradation and

stability, a key feature of exosomes as carriers of protected cargo[88]. Exosomes also contain lipid metabolism enzymes, such as phospholipases A2 and D[89], as well as enzymes necessary for lipogenesis, including fatty acid synthase, palmitate and stearate[90,91]. Finally, exosomes can also transport bioactive lipids to recipient cells, such as arachidonic acid, prostaglandin E_2, ceramide, and phosphatidic acid[86,89].

The protein content of exosomes is quite diverse and is perhaps the most well-studied exosomal cargo component. Exosome protein can vary widely depending on cell type and biological context. There are two major categories of exosomal proteins, those present on the membrane and those encapsulated within the lumen. Proteins present on exosome membranes include major histocompatibility complex class II (MHC class II) and tetraspanins (most commonly CD9, CD81, and CD63)[40,92,93]. Exosomes also contain several proteins required for MVB biogenesis and release, including the ESCRT proteins, Alix, TSG101, integrins, and chaperones such as Hcs70 and Hsp90[94]. Since these proteins are involved in the general process of exosome formation, they are often used as exosome-specific markers to indicate vesicle purity after isolation. The protein content of exosomes, which can include receptors, transcription factors, and enzymes, is often functional and can have marked effects on recipient cells after uptake[44]. For example, glioblastoma cells could successfully transport the oncogenic variant of epidermal growth factor (EGFRvIII) via exosomes to activate the MAPK/Akt proliferation cascade in neighboring glioma cells[95]. Overall, exosomal proteins are important mediators of intercellular signaling processes.

Almost every known species of RNA has been found within exosomes. As expected, mRNA and miRNA were the first to be characterized[14,96], but subsequent studies have demonstrated the presence of rRNA, tRNA, siRNA, snoRNA, scRNA, snRNA, lncRNA, and piRNA[97,98]. RNA species found in exosomes are usually smaller in size than those found in cellular fractions, which is logical due to the small size of nanoparticles[98]. The concentration of RNA in exosomes has been found to vary based on the cell of origin, for instance several types of cancer cell exosomes have been shown to contain significantly more RNA than normal cells[49]. Of note, while exosomal RNA does to a large degree reflect the RNA profile of the cell of origin, some RNAs have been observed to be enriched in exosomes[14,96]. Due to the enrichment of certain RNA species in exosomes, there have been some investigations into potential selective exosomal RNA packaging mechanisms[99–101]. Several studies have implicated exosomes in the transfer of functional RNA to recipient cells. Recipient cell translation assays have shown that mRNAs delivered by exosomes can be successfully translated[14,96,102]. Additionally, microRNAs carried by exosomes could be involved in the regulation of mRNA translation in recipient cells[103,104]. Finally, advances in therapeutic applications of exosomes have led to the development of exosomes loaded with specific RNA species for targeted treatment. In a major study, Kamerkar et al. generated "iExosomes," which contained siRNA specific to the oncogenic KRAS variant KRAS^{G12D}. Treatment with the engineered iExosomes suppressed tumors in pancreatic cancer mouse models and demonstrated a specific, targeted approach for effective cancer therapy[51]. These observations demonstrate

that exosomal RNA has functional consequences in recipient cells, and that it could potentially be utilized as targeted therapy for previously difficult to treat diseases.

Easily the least characterized of the major exosomal cargo components, DNA is also possibly the molecule with the most diagnostic and therapeutic potential. Guescini et al. first reported the presence of mitochondrial DNA species within exosomes in 2010[11], followed a year later by Balaj et al.'s discovery of single stranded DNA in human and mouse glioblastoma exosomes[49]. Our laboratory was the first to identify double-stranded DNA species in exosomes using PDAC patient serum samples as well as immortalized cell lines[46]. Results from this study also determined that when pooled, the exosomal DNA was able to be sequenced and spanned the entire genome, and it also reflected the mutational status of the patient tumor or cells of origin[46]. This study was the first to implicate the diagnostic potential of exosomes isolated from patient body fluids for DNA-based mutational characterization, diagnostics, and treatment monitoring, but a handful of others have since corroborated those findings[47,105–107]. Since the membranes of exosomes prevent their contents from degrading, exosomal DNA is protected from endogenous DNase activity that affects other extracellular biomarkers such as circulating cell-free DNA, for example. Evidence supporting this notion is seen in the relatively large fragment sizes of DNA that are able to be isolated from exosomes, and their ability to be reliably sequenced[46]. Accordingly, other groups showed reliable detection of BRAF and EGFR mutations in melanoma-derived exosomes[47].

There are still many mysteries surrounding exosomal DNA that remain to be resolved. The relative scarcity of exosome DNA-related studies has contributed to the

slow accumulation of information regarding the origin, packaging, and function of exosomal DNA. A study by Takahashi et al. proposed that exosomal DNA may serve as a mechanism of cellular homeostasis by removing ectopic DNA fragments from the cytoplasm and preventing the activation of the cytosolic DNA-sensing stimulator of interferon genes (STING) pathway[108]. Work from Kitai et al. also showed that topotecan treatment significantly increased exosomal DNA production and led to dendritic cell activation through the STING pathway in breast cancer cells[109]. Another group proposed an entirely different role for exosome DNA, demonstrating that it could be delivered to recipient cells, localized to the nucleus, and was potentially transcribed[110,111]. Indeed, horizontal transfer of DNA via exosomes has been shown in model organisms[112], and altered phenotypes in recipient cells have been attributed to the delivery of exosomal DNA[48,113]. These studies demonstrate that while some progress has been made in terms of the characterization of exosome DNA, much remains to be discovered. The limited amount of information available regarding exosomal DNA, especially in comparison with the other exosome cargo components, reveals a fundamentally broad lack of understanding in the field.

Biological Functions of Exosomes

Exosomes are diverse, heterogeneous nanoparticles which have been implicated in a variety of biological processes. The function and cargo of exosomes depends on the status and type of the cell of origin, as they have been shown to be released from virtually all cell types during both normal physiological conditions and at times of stress. Initially, exosomes were thought to be purely waste removal vehicles with no additional biological activity or relevance[40]. However in the years

since the first exosomes were isolated, they have been demonstrated to have important roles in a wide variety of biological applications, serving as carriers of intercellular signals via their diverse cargo. Exosomes can successfully transfer proteins, enzymes, lipids, RNA, and even DNA to recipient cells both locally and systemically, and therefore are involved in many physiological and pathological processes[65].

The first indication that exosomes could perform distinct biological functions came from the seminal study by Raposo et al., which showed that B cell exosomes could present MHC class II peptide complexes to T cells and induce an immune response[8]. Subsequently, it has been discovered that exosomes have a variety of immunomodulatory functions. Dendritic cells, for example, were also found to release T-cell activating exosomes that were so effective they could reduce tumor burdens in mice[7,114]. Surface bound macrophage-derived exosomes have also been shown to have the ability to present antigens to T cells[115]. Additionally, there is even evidence that exosomes released by immune cells can mediate antigen presentation, inducing immune responses on their own[116]. Conversely, regulatory immune cells (Tregs) also release exosomes that are able to decrease immunogenicity by preventing CD4+ T cells from activation and inducing tolerogenic dendritic cells[117,118]. The immunosuppressive abilities of Treg exosomes have been attributed to a variety of transported cargo, including miRNAs, chemokines, and interleukins[117]. Exosomes from immunosuppressive mesenchymal stem cells (MSCs) have been observed to inhibit macrophage responsiveness by causing impaired recognition of Toll-Like Receptors (TLRs)[119], and have also been shown to promote Treg differentiation[120].

Furthermore, exosomes have been observed to facilitate cytokine delivery at immune synapses between T cells and antigen presenting cells (APCs), serving to propagate immunomodulatory signals between cell types[121]. Exosomes have many established roles in immune response signaling, with more to be discovered. Current research seeks to further clarify the roles of exosomes in immune signaling, as well as explore their potential utility for immunotherapy applications.

In addition to their abilities to activate or suppress immune cells, exosomes have become known as potent mediators of inflammation. Circulating exosomes demonstrate the ability to induce the release of several pro-inflammatory cytokines, such as interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα) and chemokine C-C motif ligand 2 (CCL2)[122]. Exosomes have also been implicated in the pro-inflammatory processes of several diseases, including cancer[123], inflammatory bowel disease[124], diabetes[125], obesity[126], and rheumatoid arthritis[127]. The mediation of neuroinflammation in several neurodegenerative diseases, including Alzheimer's and Parkinson's, has also been linked to exosomes containing factors such as α-synuclein, amyloid β, and prions[128]. Work investigating the exosomes released during the pathogenesis of chronic inflammatory states includes attempts to identify novel exosome-associated biomarkers that could be used for diagnosis and treatment monitoring of inflammation-mediated diseases[129].

Another biological function linked to exosomes is apoptosis, an important process for both healthy and diseased cells involving programmed cell death. It has been well established that dying cells release large microvesicles, called apoptotic bodies, that can be up to several microns in diameter[130]. More recently, the smaller

microvesicle population of exosomes has also been associated with apoptosis-related signaling and functions. Stromal cell-derived exosomes, for instance, have been observed to release anti-apoptotic signals to support the survival of neighboring tumor cells[131]. Additionally, cardiac progenitor cells (CPCs) can release exosomes that protect cardiomyocytes from oxidative stress by preventing ischemia- and reperfusion-induced apoptosis[132]. Placental cells of the syncytiotrophoblast constitutively release exosomes into the bloodstream that inhibit the function of the maternal immune system and promote fetal survival through their high levels of pro-apoptotic molecules such as Fas ligand (FasL), TRAIL, and PD-L1[133]. Cancer cell-derived exosomes have also exhibited immunosuppressive and pro-tumorigenic capabilities through the induction of T cell apoptosis via delivery of FasL[134]. The ability of exosomes to mediate both pro- and anti-apoptotic cell fates demonstrates their importance as a mechanism of maintaining cellular homeostasis, the disruption of which can lead to pathogenic consequences.

In addition to apoptosis, exosomes are also involved in signaling pathways related to cell proliferation. Exosomes have been shown to induce proliferation in a variety of cell types, including T cells[8], stem cells[135], and especially cancer cells[95,136,137]. The capability of exosomes to help promote and sustain proliferative signaling is the subject of intensive study in the cancer biology field. Modulation of exosome proliferative signaling, whether through the inhibition of exosome release or the manipulation of their cargo, is a potential therapeutic avenue for the inhibition of tumorigenesis[138]. Additionally, some groups have investigated the potential utility of

exosome proliferative signaling for stem cell regeneration[139,140]. Thus, cell proliferation signaling is one of the most therapeutically relevant biological functions of exosomes.

Another similar function of exosomes is the mediation of angiogenesis, or the formation of new capillaries from existing blood vessels[141]. T lymphocytes, which closely interact with vascular endothelial cells during circulation and trans-endothelial migration, have demonstrated the ability to alter VEGF signaling and tube formation through exosomes[142]. Trophoblast cells generate extracellular matrix metalloproteinase inducer (EMMPRIN)-containing exosomes that have been implicated in placental angiogenesis and remodeling[143,144]. Additionally, exosomes isolated from mesenchymal stem cells (MSCs) have been shown to enhance angiogenesis during skin repair after severe burns[145]. The bulk of the research done on exosome-mediated angiogenesis, however, has occurred in cancer-related contexts, as one of the hallmarks of cancer is the aberrant formation of new vasculature to provide nutrients to growing tumors[146,147]. For example, it has been shown that cancer cell derived exosomes taken up by endothelial cells promote angiogenesis via induction of the pro-angiogenic secretome[32,148]. Additionally, melanoma cell exosomes have been found to promote metastasis by recruiting circulating melanoma cells to the endothelium, while simultaneously releasing pro-angiogenic and extracellular matrix remodeling factors to encourage tumor formation[149]. Tumor exosomes were also shown to transfer activated EGFR to endothelial cells, leading to the induction of VEGF expression and subsequent stimulation of angiogenesis[15]. Exosomes derived from cancer cells have also demonstrated the ability to impair the structural integrity of endothelial cells by

downregulating tight junction proteins such as ZO-1, enhancing vascular permeability and thereby facilitating metastasis[150]. Since angiogenesis is so closely associated with various pro-tumorigenic and metastatic mechanisms, such as proliferation, migration, and extravasation[151], the influence of exosome-mediated signaling on angiogenic processes are being investigated as potential avenues for therapeutic intervention against metastatic dissemination and tumor formation.

There is substantial evidence for the importance of exosomes in a wide variety of biological functions. The roles of exosomes described here, while extensive, are by no means completely comprehensive. Exosomes are beginning to be implicated in even more areas, such as DNA damage response[152] and metabolism[153]. These diverse capabilities underscore the importance of exosomes in virtually all biological signaling pathways and highlight the vast potential for the development of exosome-based therapeutics.

Exosomes in Cancer

Exosomes have been implicated in several processes associated with tumor progression and metastasis, and cancer-related research is a major field of exosome work, due to the enormous potential for exosomes in diagnostics and therapeutics[154,155]. Since exosomes are so readily available in easily accessible biofluids such as serum and urine, they have been investigated for their utility for liquid biopsy[23,156]. Exosome release has been shown to be upregulated in several cancer types, including breast, ovarian, and pancreatic cancer[157,158]. Thus far, relatively few cancer exosome-specific markers have been identified, with notable exceptions being the glypican-1 (GPC-1) molecule in breast cancer cell lines[158], MET in melanoma[32],

and macrophage migration inhibitory factor (MIF) in pancreatic cancer[159]. Strides have been made, however, in utilizing the cargo of exosomes, specifically RNA and DNA, to detect oncogenic variations in patients. Since the work done initially by our lab demonstrated that exosomal DNA can be sequenced and faithfully recapitulates the mutational landscape of the cell of origin[46], several studies have utilized similar techniques to identify markers of disease. A few RNA species contained in exosomes have been identified as potential biomarkers, such as the mRNA for oncogenic EGFRvIII in glioblastoma[137] and miR-21 in esophageal squamous cell carcinoma patients[160]. Additionally, the levels of miR-141 isolated from the exosomes of prostate cancer patients were able to be used to accurately distinguish between patients with metastatic vs. localized disease[161]. Perhaps even more promising is the use of exosomal DNA to identify cancer-specific mutations. We recently published a study in which we were able to successfully identify mutant KRAS and TP53 in PDAC patient serum exosomes[162]. Additionally, we were able to distinguish between PDAC patients, healthy donors, and chronic pancreatitis patients based on the mutations detected using exosomal DNA[162]. Other studies have demonstrated similar findings using serum-derived exosomes[47,106], and exosomal DNA is beginning to be investigated further as a means of early diagnosis for a variety of cancers.

Beyond their use as potential biomarkers, exosomes have been demonstrated to perform several functional roles in tumor progression. As previously mentioned, exosomes have many roles in the regulation of adaptive immunity. Immunogenic activity has also been identified for exosomes in both pro- and anti-tumorigenic contexts. For example, tumor-derived exosomes could possibly serve as a source of

tumor antigens for presentation to activated T cells[163–165]. Additionally, cancer exosomes have been observed to activate NK cells through the presentation of HSP70[166]. Subcapsular lymph node macrophages have also been observed to absorb tumor-derived exosomes, preventing them from interacting with pro-tumorigenic B cells and thus limiting tumor progression[167]. On the other hand, exosomes have also been implicated in tumor immune evasion. Exosomes can inhibit dendritic cell maturation by inducing IL-6 expression in dendritic precursors[168]. Additionally, cancer cell-derived exosomes demonstrate the ability to inhibit NK cell proliferation and cytotoxic function[169], induce T cell apoptosis via FasL[134], and suppress T cell receptor (TCR) activity[170,171]. TGFβ1 carried by tumor exosomes has also been shown to induce Tregs[172]. Finally, exosomes from primary glioblastoma cells were shown to influence monocyte-to-macrophage differentiation to promote a tumor-supportive phenotype *in vitro*[173]. Combined, these findings illustrate the multifaceted behavior of exosomes in tumor-related immune responses. Once a more comprehensive understanding of the immunomodulatory functions of exosomes in cancer is established, they may become useful in immunotherapy applications. Indeed, some clinical trials have already begun incorporating exosomes as treatments to activate antitumor immune responses[174].

Exosomes also induce pathological angiogenesis in many cancers. As cancer progression occurs, tumors outgrow the existing vascular network, generating hypoxic regions, which in turn leads to the release of neovascularization-stimulating factors packaged in exosomes. Endothelial cell uptake of cancer-derived exosomes has been shown to promote angiogenesis and new blood vessel formation[15,175–177].

Exosomes released from hypoxic tumors have also been demonstrated to increase activation of the ERK1/2 MAPK, PI3K/AKT, and FAK pathways in recipient endothelial cells, leading to increased endothelial cell sprouting[148]. In addition to endothelial cell proliferation and new tube formation, cancer cell exosomes may also promote vascular remodeling through the manipulation of blood vessel wall permeability, facilitating metastasis. Melanoma exosomes have been observed to induce vascular leakiness at sites of metastasis[32], and the delivery of exosomal miR-105 to endothelial cells downregulates ZO-1, a tight junction protein, enabling vessel permeabilization and metastatic dissemination[150].

Cancer cell-derived exosomes can also mediate signaling between tumors and cells in the surrounding microenvironment. Notably, cancer exosomes show the ability to activate fibroblasts to generate a more tumor-permissive state[178]. The stimulation of cancer-associated fibroblasts (CAFs) by tumor exosomes promotes the acquisition of pro-tumorigenic properties, such as the induction of myofibroblast-like phenotypes in mesenchymal stem cells[179] and myofibroblast differentiation[180]. Myofibroblasts are activated fibroblasts that express alpha smooth muscle actin (α -SMA) and are much more abundant in tumor microenvironments than in normal tissues, so their presence is generally indicative of a pro-tumorigenic environment. CAF-derived exosomes have also been discovered to have pro-tumorigenic properties, as they have been implicated in mediating therapy resistance in cancer cells[181,182]. Exosomes could also play a role in tumor-driven extracellular matrix (ECM) remodeling in the tumor microenvironment, as they have been associated with enhanced motility and invasiveness through their ability to bind to individual ECM components and propagate

protease-mediated degradation of ECM components such as collagen and fibronectin[91,183].

Tumor-derived exosomes are involved in a number of metastasis-promoting functions beyond those previously mentioned for angiogenesis and tissue remodeling. Since exosomes are capable of traveling systemically throughout the body, it has been proposed that they may have the ability to 'prime' pre-metastatic niches at distant sites and enable the recruitment and successful colonization of new tumors. David Lyden's group has published several studies indicating exosome-mediated initiation of pre-metastatic niches in several cancer types, including melanoma, pancreatic cancer, and breast cancer[32,159,184]. Additionally, they demonstrated through the use of cell lines with preferential tropism for certain metastatic sites that exosomes isolated from each line, when injected *in vivo*, would fuse preferentially with cells at their predicted destinations, providing evidence for exosomes serving as organotropic mediators of pre-metastatic niche formation[184]. Exosomes have also been shown to enhance the invasion and colonization capacities of tumor cells themselves, acting through delivery of cargo components such as miR-200[185] and Wnt11[186]. The inhibition of exosome secretion *in vivo* was also shown to decrease the speed and directionality of cancer cell migration in chick embryos[187], highlighting the importance of exosome-mediated signaling for cell motility. Exosome transfer from highly invasive cells to less malignant recipients also led to the increased migration of recipient cells in mouse xenografts at both local and distant sites[155]. Finally, exosomes have been shown to be capable of inducing an epithelial-to-mesenchymal transition (EMT) in target cells, facilitating

migration[188]. Exosomes have clearly been shown to have the capacity to influence metastasis through a variety of different biological pathways and processes.

Lastly, exosomes have repeatedly been shown to contribute to tumor growth and progression. Exosomes from non-tumorigenic cells can be co-opted into tumor supporting roles; for instance, exosomes derived from astrocytes have been demonstrated to support tumor growth for brain metastases by targeting the tumor suppressor PTEN with miR-19a[189]. Exosomes can also serve as a delivery vehicle for the oncogenic variant of the epidermal growth factor receptor (EGFR), known as EGFRvIII, as seen in glioma cells, leading to enhanced proliferation[95]. Glioblastoma exosomes were similarly discovered to contain RNA and proteins that promote tumor growth upon their delivery to recipient cells[137]. Additionally, ovarian carcinoma derived exosomes enhanced tumor growth when delivered to tumor-bearing mice[136]. Exosome involvement in cancer initiation, growth, migration, and survival has been definitively demonstrated in a variety of organisms and tumor types, which implies that exosomes are not only integral to cancer signaling and survival but may also serve as potent mediators for therapeutic interventions.

Diagnostic and Therapeutic Applications of Exosomes

Since exosomes are released by virtually all cell types and are also involved in the progression of many different diseases, it stands to reason that they would be the subject of great interest for the development of therapeutics. Naturally, many research groups and biotechnology firms have worked on developing exosome-based diagnostics and treatments. Described here are some of the major clinical applications

of exosomes for disease intervention, including their use as biomarkers, therapeutic targets, and therapeutic agents.

Due to their heterogeneous cargo that has been shown to reflect characteristics of their cells of origin, exosomes have repeatedly been investigated as biomarkers for disease diagnosis, progression, and treatment response. Since large quantities of exosomes are present in biofluids, they have the advantage of being a sensitive and relatively non-invasive method to detect tumors and other indicators of disease. Cancer exosomes in particular have been demonstrated to be useful for the detection of many types of tumors, including prostate, breast, ovarian, glioblastoma, and melanoma[137,190–193]. Our lab demonstrated the utility of exosomal DNA for the detection of cancer-specific mutations from PDAC patient serum[158,162], work that was later validated by others[105–107]. Additionally, exosomal RNA species have been identified as viable cancer progression markers[104]. Exosomes have been useful biomarkers for other diseases as well. Exosomes contain misfolded proteins associated with neurodegenerative disorders, and the detection of β-amyloid 42 and tau proteins in Alzheimer's patients was possible in cerebrospinal fluid exosomes at an early stage of the disease[194,195]. Exosomes isolated from urine have also been shown to indicate various types of kidney disease, including renal ischemia and reperfusion, nephrotic syndrome, and acute kidney injury[196–198]. Exosomes have even been used to identify complicated pregnancies using levels of circulating placental exosomes expressing FasL[199]. Serum exosomes have also been shown to be markers for infectious diseases, such as tuberculosis[200] and HIV[201]. Exosomes and their cargo continue to be investigated as potential biomarkers, and will likely continue to be

relevant for years to come through their utility for applications such as PCR amplification, sequencing, and proteomics[202].

Exosomes can also be utilized as therapeutic targets for the treatment of disease. Since exosomes are involved in so many pathological conditions and processes, it stands to reason that disease progression could potentially be halted by specifically inhibiting exosome production or release, or by inhibiting their uptake. Inhibition of ceramide formation, an important step in exosome biogenesis, using small molecule inhibitors of sphingomyelinase or treatment with the drug amiloride, which attenuates endocytic vesicle recycling, can inhibit the release of exosomes[62,203]. Amiloride treatment has been shown to inhibit tumor growth *in vivo* by blocking the secretion of tumor-derived exosomes[203]. In some tumor cells, exosome release is dependent on the small GTPase RAB27A, which can be targeted by RNAi to reduce tumorigenic exosome signaling and thereby reduce the growth rate and metastasis of tumors[70,74]. The uptake of exosomes by recipient cells can be inhibited by blocking surface phosphatidylserine using annexin V[204]. This strategy reduced the growth of human glioma xenografts in mice. Additionally, blocking specific signaling components of exosomes has been shown to have some therapeutic efficacy. Monoclonal antibodies targeting FasL1 on the membranes of exosomes reduced melanoma tumor growth[205–207]. One major caveat to consider is that interfering with exosome biogenesis could result in off-target effects, since exosomes have important functions in normal biological processes.

Exosomes are also being investigated for use as therapeutic agents. In regenerative medicine, MSC exosomes have been utilized to improve heart function

after myocardial ischemia-reperfusion injury[208], as well as in a similar model of kidney disease[209]. Exosomes have been exploited for immunotherapy due to their multitude of immunomodulatory functions. The use of exosomes for this application dates back to Raposo et al.'s 1996 study, in which MHC Class II bearing exosomes were shown to induce T cell responses and subsequent tumor growth suppression[8]. Exosomes are also now being used for targeted drug delivery. Since exosomes have been demonstrated to transfer functional content, such as RNA and protein, to recipient cells, it follows that loading exosomes with a therapeutic molecule could serve as an effective delivery and treatment method. Since exosomes are endogenous and biocompatible, they are often not detected and cleared as quickly as other synthetic delivery vehicles, giving them an advantage. They can also be derived directly from the patient in a non-invasive manner, and are small enough to penetrate major biological barriers, including the blood brain barrier[41]. Exogenous siRNA was first delivered via vesicles targeted to the brain in mice, with limited observed toxicity or immune stimulation[210]. Several studies since then have demonstrated the successful use of exosomal RNA for therapeutic benefit. Our lab, for example, demonstrated the robust anti-tumor activity of exosome-delivered siRNA for the oncogenic variant $KRAS^{G12D}$ in mice with PDAC[51]. Exosomes are currently being used in an ever-increasing number of diagnostic and therapeutic applications. As more is discovered regarding their biogenesis and mechanisms of action, exosomes will be increasingly utilized for treatment interventions across a wide spectrum of diseases.

Methods for Exosome Isolation and Characterization

Description of Methods Used to Isolate and Characterize Exosomes

As exosomes are being implicated in an increasing number of biological processes and utilized more often in therapeutics, the methods of exosome isolation and characterization have evolved. The isolation of pure populations of exosomes is important for understanding their mechanisms of action and for downstream applications. Since exosomes are extremely small, collecting them is no trivial task. Newer isolation methods have attempted to address common issues observed with obtaining exosomes, such as purity and low yield or quality. Our ability to detect, characterize, and track exosomes has also improved, as established exosome markers can now be sensed on a single vesicle level for applications in flow cytometry and imaging. The isolation of exosomes was for many years dependent on high speed ultracentrifugation as the most common and reliable method for obtaining exosomes. A seminal paper published by Théry's group in 2006 served as the gold standard for the field for over a decade, and indeed is still utilized in many studies today[211]. However, spinning media at such high speeds pellets several other components in addition to exosomes, including protein aggregates and cell free DNA and RNA, that could conceivably interfere with experimental results depending on the desired output.

Thus, in recent years several other methods have gained traction as viable exosome isolation techniques, including density gradient fractionation, size exclusion, and immunoaffinity capture, among others[212]. Each method has associated benefits and drawbacks that must be taken into account when planning to isolate exosomes, and several of the methods are so novel that they still require more rigorous characterization[213]. Controversy also exists in the exosome field regarding which isolation method is superior, especially for the isolation of specific exosome cargo[214].

A portion of this work aims to clarify the landscape of exosome isolation methods, specifically relative to exosomal DNA extraction. What follows are descriptions of the different established methods for deriving exosomes from biofluids, including an analysis of what is beneficial and detrimental for each. Additionally, methods of exosome characterization and quantification are described in detail.

Ultracentrifugation

By far the most commonly used exosomal isolation technique over the past decade is high speed ultracentrifugation, still considered the gold standard[214]. While some protocol deviations may occur based on user preference and the desired readout, such as spin time and additional wash steps, the general process is relatively simple and easily replicable. First, samples are spun at low speeds to pellet any live or dead cells (300g for live cells and subsequently 2000g for dead). The supernatant is then sterile filtered with a 0.22-micron pore filter to remove any larger nanovesicles, such as apoptotic bodies. Ultracentrifugation of the supernatant then occurs at 100,000g for varying lengths of time; serum and other biological fluids are often spun longer, even overnight, because biological samples are often more viscous than cell culture samples and thus take longer to sediment. The exosome pellet is then resuspended in PBS[211]. Ultracentrifugation is the best method for exosome isolation from sources with large volumes, such as cell culture media, and provides the highest yield of recovered exosomes. Ultracentrifugation is also easy, requires little technical expertise, and involves very little sample pretreatment. Additionally, no extra chemicals or reagents are used in this method, reducing the risk of contamination and preventing any potential chemically induced damage to the exosomes. However,
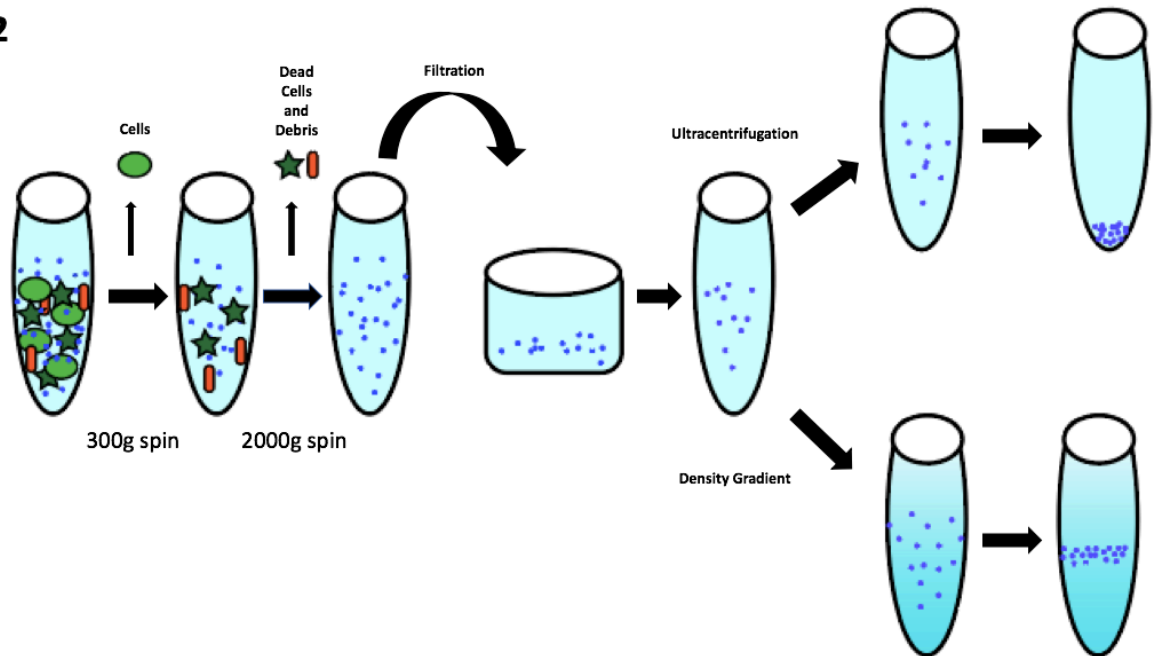
Figure 2. Schematic of ultracentrifugation-based methods.

ultracentrifugation requires very expensive machinery to run, spins are very time intensive, and it has been proposed that high speed centrifugation may actually impact exosome integrity[202,214,215]. The pellet obtained after ultracentrifugation also contains everything left in the media, not just exosomes, including other molecules such as protein, RNA, and DNA, that can confound results[213].

Density Gradients

Variations of ultracentrifugation exist, and density gradient fractionation has become a popular method to collect more highly purified exosome populations by separating particles by their density[216,217]. In density gradient fractionation, exosomes are separated by their size, mass, and density when spun in a pre-constructed density

gradient medium. To create the gradient, fractions with different densities are layered on top of each other in a continuous or discontinuous gradient in order, with the highest-density fraction on the bottom and the lowest density fraction on the top. Samples are usually layered on top of the completed gradient in an ultracentrifuge tube, but some groups have bottom-loaded the sample and layered the gradient on top[214,218]. The gradient is then spun at 100,000g, and the exosomes and other molecules contained in the samples move through the density gradient at a specific sedimentation rate. This leads to discrete zones that contain materials of varying densities. Exosomes can be recovered via simple fractionation once the spin is complete. While the purity of exosomes isolated by this method is very high, the yield is among the lowest of all exosome recovery methods. This method also takes a significant amount of time, because the gradients must be spun for many hours to fractionate the input material. There is also a limited volume of input sample that can be loaded onto the gradients, which makes large volume isolation impossible.

There are two types of density gradient ultracentrifugation, isopycnic and moving zone. In isopycnic ultracentrifugation, the medium used to create the density gradient encompasses the entire range of densities of the particles in the loaded samples. The separation of exosomes from the other particles into a discrete zone depends completely on their density difference from other particles, assuming the samples are spun for an adequate amount of time. During the spin, exosomes arrest in the gradient where they have the same density as the medium-at the isopycnic position. Once the isopycnic position has been reached, further centrifugation serves to force the exosomes into a sharper zone, but they maintain their position in the

gradient. For moving-zone ultracentrifugation, the medium used for the gradient has a lower density than any of the particles in the loaded sample. The exosomes in these gradients are separated by size and mass rather than density. This allows vesicles of similar densities to be separated based on how big they are. Because the particles are denser than the gradient medium, moving-zone ultracentrifugation is isodynamic rather than static, meaning the solutes will eventually all pellet at the bottom of the centrifuge tube if the centrifugation period is too long. To prevent exosomes from pelleting out, sometimes a high density "cushion" is layered at the bottom of the tube. After the gradient centrifugation step, samples are then resuspended in PBS and subjected to another round of ultracentrifugation at 100,000g to eliminate any remaining gradient medium[202,214]. Commonly used examples of gradient medium substrates are OptiPrep (iodixanol) and sucrose[202,218].

Table 1. Exosome Isolation Methods

Isolation Method	Mechanism	Advantages	Disadvantages
Ultracentrifugation	sequential; density, size and shape based / separates particulates from solution	large input volume capacity, high exosome yields, no contamination risk with reagents	may damage exosomes due to high speed, not pure due to co-precipitation of other molecules, high equipment cost, long run time, labor intensive
Density Gradients	separates based on size, mass, and density	high purity, substrates such as optiprep or sucrose are gentler on exosomes at high speeds	extremely low yield, low input volume, extremely time intensive; still incorporates high speed UC so exosome damage can still occur
Size-Based	exclusively based on size	Ultrafiltration: fast, no additional equipment necessary, low cost / SEC: high purity exosomes, gravity elution the gentlest method, high preservation of exosome integrity, moderate sample capacity, highly reproducible	Ultrafiltration: some shear stress-induced damage, possibility of vesicle trapping/clogging in the membrane, lower yields / SEC: equipment cost, more difficult to scale up
Immunoaffinity	based on the interaction between membrane bound receptors (antigens) of exosomes and immobilized antibodies	Superior for isolation of specific exosomes-high purity	high reagent cost, must establish and optimize exosomes markers, samples must be pure since heterogeneity impacts efficiency, susceptible to epitope being masked or blocked
Microfluidics	microscale isolation based on exosome properties such as immunoaffinity, size, density, or charge	fast, low—cost, easy automation and integration	lack of standardization and method evaluation, moderate to low sample capacity
Reagents	precipitates based on altering solubility or dispersability	straightforward, no special equipment required, scalable, low cost	High co-precipitation of non-exosome contaminating factors, potential for reagent to damage exosomes

Size-Based Techniques

Another exosome isolation method that has gained popularity recently is size-exclusion chromatography (SEC). This technique separates exosomes based on size due to their differential passage through physical barriers using filters or chromatography columns. Column chromatography allows for the sequential elution of vesicle fractions of different sizes using a single column[202]. SEC is often used in combination with ultracentrifugation to enrich the exosome yield[219]. SEC has been shown to yield very pure populations of exosomes, and by separating the fractions using gravity instead of high speed the integrity and biological activity of the exosomes is highly preserved. While SEC allows for moderate sample capacity, it cannot accommodate large volumes of sample; often, high capacity samples must be ultracentrifuged or ultrafiltered prior to SEC to obtain a feasible sample volume.

Another popular size-based exosome isolation method is ultrafiltration (UF). UF is very similar to conventional membrane filtration in that the separation of particles is primarily dependent on their size or molecular weight. Exosomes can be isolated based on their size by the selection of an appropriate membrane filter,[220] and exosome loss is minimal compared with other isolation methods[221]. UF is faster than ultracentrifugation and does not require expensive machinery,[213] however it has been suggested that the force used may damage larger vesicles and compromise the integrity of the exosomes[222]. Additionally, it is difficult to remove contaminating proteins from the samples. It has also been shown that UF membranes can become clogged and trap vesicles, decreasing the isolation efficiency[223,224]. Size-based kits for exosome isolation have also been developed. Many of these kits utilize syringe-filter

based fractionation to separate vesicle populations by their size. Sequential filtration of samples using membranes with different pore sizes to filter out soluble populations of certain sizes has additionally been implemented for the isolation of exosomes.

Assymetric flow-field-flow fractionation (AF4) is also used to isolate exosomes on the basis of size. AF4 utilizes a porous rectangular channel across which a sample is carried via parabolic flow. A crossflow across the channel controls sample retention and distributes vesicle components against the channel wall based on the diffusivity of their components[225]. Smaller particles diffuse further from the accumulation wall and are eluted earlier than large ones. This method is more scalable since it can rapidly isolate exosomes and input fluid can easily be added in larger volumes, though not at as high a capacity as with ultracentrifugation[226,227].

Immunoaffinity

The membrane-bound proteins and receptors on the surface of exosomes provide many opportunities for the development of immunoaffinitive mechanisms of isolation, whether through proteins and antibodies or receptors and their ligands. A microplate-based enzyme-linked immunosorbent assay (ELISA) was developed to specifically derive exosomes from serum, plasma, and urine. Absorbance assays can provide a comparison of the expression levels of known surface exosome biomarkers, as well as quantify the captured exosomes. These assays have been shown to produce comparable results to ultracentrifugation but with much less sample volume, demonstrating superior specificity and yield, which is extremely important for precious small volume samples such as those obtained from patients[228]. In a similar application, submicron-size magnetic particles were combined with immunoaffinity to generate a

technique termed magneto-immunocapture. It was found that with just 1 mL of cell culture supernatant, antibody-coated magnetic beads were able to capture as many exosomes as with large volume ultracentrifugation[228].

Immunoaffinity methods are rapid, easy to use, and work with general benchtop equipment. Commercial kits are now available to isolate exosomes based on the concept of magneto-immunocapture using common exosome surface markers, such as the tetraspanins. These methods, however, require a degree of washing and pre-enrichment to enhance the quality of the sample prior to isolation. When compared to ELISA assays, however, immunoaffinity capture with magnetic beads has been demonstrated to have a higher capture efficiency and better sensitivity. Additionally, bead-based assays can be easily scaled up or down for any volume of sample. Immunoaffinity capture was also recently combined with mass spectrometry to capture exosomes with antibodies immobilized on porous monolithic silica micropipette tips in a technique called the mass spectrometric immunoassay[229]. In this automated assay, a multichannel pipette system enables the isolation of exosomes from up to 12 samples at once.

While immunoaffinity assays generally offer superior isolation of specific and highly purified exosomes, they are extremely expensive. Exosome tags also must be established and optimized, and the low input capacity means that the exosome yield is low. Minor heterogeneity in the exosome population can also impact effective immunocapture, and there is also the risk that the antigenic epitopes on the exosome surface might be blocked or masked[220,230].

Microfluidics

Microfluidics-based devices are now being used to quickly and efficiently isolate exosomes using their physical and biochemical properties. In addition to traditional isolation characteristics such as size, density, and immunoaffinity, microfluidic devices can also incorporate acoustic[231], electrophoretic, and electromagnetic manipulations[232]. These devices have been shown to decrease the amount of sample volume required to yield a given number of exosomes, thereby decreasing reagent consumption and isolation time. To enhance the specificity of exosome capture and introduce the capability to simultaneously subtype captured exosomes, immunoaffinity capture was integrated with a microfluidic chip to isolate specific exosome populations[233]. Antibodies to specific exosome membrane-bound proteins were immobilized on a chip, enabling exosome isolation from the sample input. A commercialized product called ExoChip is also commercially available and relies on microfluidic technology to isolate CD63 positive exosomes using an immunochip containing anti-CD63. Exosomes are stained with carbocyanine dye (DiO), allowing for quantification of captured exosomes with a plate reader[234]. Additional microfluidic devices are being developed to potentially capture and sort exosomes based on properties such as charge and marker expression. In addition to moderate to low sample capacity, however, a general lack of both method validation and standardization have hampered the use and acceptance of microfluidics for exosome collection[235].

Reagents

Exosomes can be precipitated out of biological fluids or media using reagents that alter their solubility or dispersibility, such as water-excluding polymers like polyethylene glycol (PEG)[213]. The water-excluding polymers engage water molecules, thus forcing less soluble components out of solution. Exosome precipitation by this type of method is very straightforward and does not require any specialized equipment, which also allows for easy scalability. Many exosome precipitation kits are commercially available and work with a variety of fluids such as cell culture media, serum, and urine[219]. A major disadvantage of these methods is the co-precipitation of non-exosome contaminating factors, such as proteins and polymeric materials, necessitating pre- and post-isolation purification steps. These isolations also require a long incubation period.

Nanoparticle Tracking Analysis

In addition to the variety of methods available for exosome isolation, there are also several techniques that aid in the characterization and visualization of isolated nanoparticles. Nanoparticle Tracking Analysis (NTA) involves optical particle tracking, which can measure the size distribution and concentration of exosomes and particles between 10nm and 2µm in diameter. The path of particle movement is detected based on the defraction of light by particles, allowing for the tracking of Brownian motion of particles in a liquid suspension[236]. The NTA then measures the movement of the particles by tracking each one during image analysis. It is this movement that is then correlated back to determine particle size based on the Stokes-Einstein equation[237]. NTA has multiple advantages, including the ability to detect multiple classes of extracellular vesicles, including those as small as 30nm in diameter. Sample

preparation is also extremely easy and only requires a small input amount, saving much of the sample for other downstream applications. The samples can also be recovered from the detection chamber if desired[238]. Additionally, only a few minutes per sample are required for data collection and analysis. NTA also has the capacity to detect the presence of antigens on the surface of vesicles with the application of fluorescently labeled antibodies[236].

Dynamic Light Scattering

Photon Correlation Spectroscopy, also known as dynamic light scattering or DLS, is an alternative technique for measuring exosome size. A monochromatic laser beam is passed through a suspension of particles, and time-dependent fluctuations in scattering intensity resulting from the relative Brownian moments of the particles are observed[238]. This method does not visualize the particles, it just measures the size, but the particles can be anywhere from 1nm to 6μm in diameter. This technique works best when applied to one type of particle in a suspension, because vesicles of different sizes can lead to detection errors[239,240].

Table 2. Exosome Characterization Methods

Method	Mechanism	Exosome Properties
Nanoparticle Tracking Analysis	optical particle tracking	size distribution, concentration
Dynamic Light Scattering	Brownian motion tracking via light scattering	size
Flow Cytometry	laser based detection of light scattering, fluorescence	surface protein expression, size, structure
Transmission Electron Microscopy	electron beam excitation and detection	size, diameter, structure
Western Blots	protein detection with antibodies	protein

Flow Cytometry

Flow cytometry is one of the most frequently utilized techniques for exosome characterization and analysis. It can be used to identify exosomal surface proteins in a given population of exosomes, as well as measure exosome size and structure[241]. Conventional flow cytometers measure particles greater than 300nm in diameter but are not able to detect smaller particles such as exosomes directly. However, exosomes can be conjugated to beads that enable their detection[242]. For detection, a laser beam with a specific wavelength is directed through a stream of particles suspended in fluid. The degree of light scattering is detected and is based on the number, size, and granulation of the particles. Additionally, fluorescent antibodies for exosomal markers can be detected with flow cytometry, enabling the identification and quantification of exosome biomarkers, such as the tetraspanins, in a given exosome population. Recently, specialized flow cytometers have been developed specifically for the characterization of nanoparticles that can distinguish stained exosomes from background noise[243,244].

Transmission Electron Microscopy

Transmission Electron Microscopy, or TEM, is one of the first methods ever used to characterize exosomes[5]. It is a technique used to observe the structure, morphology, size, and location of various biological components, including exosomes. A beam of electrons is passed through a specially prepared sample, where a secondary electron is generated. These electrons are detected, collected, and magnified using special lenses. For biological samples, both TEM and cryo-electron microscopy (cryo-EM) are typically used. Specimens must be fixed in glutaraldehyde

and dehydrated, and the images must be taken under a vacuum. TEM images of vesicles can be used for measurements of size and diameter, as well as inform on the location or presence of vesicles in certain tissues or organelles. Exosomes are often visualized with immunogold-labeled antibodies specific for certain cargo components in order to characterize the contents of the exosomes. Caveats to this approach include the consideration that the extensive sample preparation process may alter vesicle morphology. Additionally, the electron beam could damage biological samples. For example, exosomes observed with TEM have a cup-shape morphology, while frozen exosomes examined with cryo-EM are round[43]. More and more studies are utilizing cryo-EM for exosome samples, as the preparation does not require the dehydration and fixing steps since they are snap frozen in liquid nitrogen and the structures remain intact.

Western Blots

One of the most common methods for evaluating exosome protein cargo components is western blotting[214]. The process is almost identical to that for cell-derived protein and, while time-consuming, is very straightforward. Western blots for canonical exosome markers such as the tetraspanins CD9, CD81, and CD63, as well as others like flotillin and TSG101, are often an integral part of any publication involving exosomes as part of exosome validation. Additionally, more sensitive assays such as those run on the ProteinSimple WES apparatus can detect protein at picogram-level sensitivity, enabling better evaluation of less abundant exosome protein components.

Dissertation Goals and Major Findings

The main goal of this work was to comprehensively characterize the DNA packaged into exosomes. We also sought to identify the best exosome isolation methods for subsequent exosomal DNA isolation and analysis. Additionally, we hoped to determine whether exosomal DNA had utility for the identification and evaluation of DNA methylation marks. Finally, we wanted to investigate the roles of exosomes in mediating DNA damage response signaling in the tumor microenvironment, and elucidate potential consequences of those effects for tumor survival.

Several major findings are described in the following pages. First, exosome DNA is heterogenous, and its packaging is dependent on the cells of origin. Additionally, after a thorough evaluation of common exosome isolation methods, we determined that size exclusion chromatography (SEC) was the superior technique with regards to exosome DNA yield and purity. We also discovered for the first time that exosomal DNA is not only methylated, but faithfully recapitulates the methylation patterns of the cell of origin, enabling the detection of site-specific methylation variations. A preliminary mechanism for DNA packaging into exosomes was identified, highlighting the role of nuclear envelope integrity in the ability of DNA to escape the nucleus to become available for incorporation into exosomes. Finally, we discovered that exosomes are paracrine signaling mediators of DNA damage response activation in treatment-naïve cells. Furthermore, we establish that exosomes from cancer cells with induced DNA damage carry activated DDR pathway proteins that could initiate DDR in treatment-naïve cells, uncovering a potential mechanism of exosome - mediated bystander DDR induction upon delivery to recipient cells.

CHAPTER 2

MATERIALS AND METHODS

Cell Line Characterization

Cell Lines and Culture Conditions

Tumorigenic and non-tumorigenic cell lines used for these studies are listed in

Table 3. Cells were grown to no more than 70-85% confluence and maintained by

Table 3. Cell Lines and Growth Conditions

Cell Line	Origin	Tissue	Type	Tumorigenic	Growth Conditions	Reference
BJ	Human	Foreskin	Fibroblast	No	DMEM, 10% FBS	(Morales et al., 1999)
BXPC3	Human	Pancreas	Epithelial	Yes	RPMI, 10% FBS	(Tan et al., 1986)
Capan C2-14	Human	Pancreas	Epithelial	Yes	DMEM, 20% FBS	(Sakai et al., 2008)
Capan-1	Human	Pancreas	Epithelial	Yes	DMEM, 20% FBS	(Fogh et al., 1977)
DiFi	Human	Colon	Epithelial	Yes	DMEM, 10% FBS	(Olive et al., 1993)
DOV13	Human	Ovary	Epithelial	Yes	RPMI, 15% FBS	(Ramakrishnan et al., 1989)
HEK-293T	Human	Kidney	Lymphocyte-Like	No	DMEM, 10% FBS	(DuBridge et al., 1987)
HK2	Human	Kidney	Epithelial	No	DMEM, 10% FBS	(Ryan et al., 1994)
HPDE	Human	Pancreas	Epithelial	No	1:1 Keratinocyte serum-free media/ DMEM, 10% FBS	(Ouyang et al., 2000)
HPNE	Human	Pancreas	Epithelial	No	RPMI, 10% FBS	(Lee et al., 2003)
MCF7	Human	Breast	Epithelial	Yes	DMEM, 10% FBS	(Brooks et al., 1973)
MCF10A	Human	Breast	Epithelial	No	1/1 DMEM/F12, 5% equine serum, 20 ng/ μL EGF, 0.5 mg/ mL hydrocortisone, 100 ng/mL cholera toxin, 10ug/mL insulin	(Soule et al., 1990; Tait et al., 1990)
MDA-MB-231	Human	Breast	Epithelial	Yes	DMEM, 10% FBS	(Young et al., 1974)
MiaPaCa2	Human	Pancreas	Epithelial	Yes	DMEM, 5% equine serum	Yunis et al (Yunis et al., 1977)
OVCAR3	Human	Ovary	Epithelial	Yes	RPMI, 20% FBS	(Hamilton et al., 1983)
OVCAR5	Human	Ovary	Epithelial	Yes	DMEM, 10% FBS	(Hamilton et al., 1984)
Panc1	Human	Pancreas	Epithelial	Yes	RPMI, 10% FBS	(Lieber et al., 1975)
PSN1	Human	Pancreas	Epithelial	Yes	RPMI, 10% Heat-Inactivated FBS	(Yamada et al., 1986)
SKOV3	Human	Ovary	Epithelial	Yes	1/1 MCDB105/ M199 1:1, 10% FBS	(Fogh et al., 1977)
SW620	Human	Colon	Epithelial	Yes	10% FBS, DMEM/ F12	(Leibovitz et al., 1976)
T3M4	Human	Pancreas	Epithelial	Yes	10% FBS RPMI	(Okabe et al., 1983)

Table References[245–265]

passage every 4-5 days. All cell lines were validated at MDACC Characterized Cell Line Core Facility and routinely checked for mycoplasma contamination using Sigma LookOut Mycoplasma PCR Detection Kit, (Cat. No. MP0035-1KT).

Viability Testing

Cells grown to 70-85% confluence were washed with phosphate buffered saline (PBS) and incubated 48 hours in the appropriate serum-free culture media with 1% BSA supplementation or complete media supplemented with FBS (**Table 3**). The cells were harvested by trypsinization and viability was measured by staining with the APC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, Cat. No. 640930) following manufacturer's instructions and was immediately analyzed by flow cytometry (BD LSRFortessa X-20).

Exosome DNA Experiments

Exosome Isolation: Differential Centrifugation (UC)

Cells cultured in T225 flasks (Falcon, Cat. No. 353138), to 70-85% confluence were washed three times with PBS. The cells were then incubated 48 hours in in serum-free media supplemented with 1% Bovine Serum Albumin (BSA). Conditioned media were centrifuged 5 min at 800G, followed by 10 min centrifugation at 10,000G, to remove cells and smaller debris. Supernatants were filtered through 0.20 μm pore filters (Corning, Cat. No. 431219) and subjected to ultracentrifugation (UC) for 3 hours at 100,000g (Beckman, SW32Ti rotor). The supernatants were discarded and exosome pellets resuspended in PBS by manual pipetting, 5 minutes per sample. Patient serum samples were spun overnight and supernatant was kept at -80°C.

Exosome Isolation: Size Exclusion Chromatography (SEC)

Exosomes collected by UC, as above (crude concentrates) were resuspended, diluted with PBS, and subjected to a wash spin (3 hours, 100,000g). Exosome pellets were resuspended in 300 μL PBS and loaded onto temperature-equilibrated qEV Size Exclusion columns (IZON qEV Original 35nm Column, Fisher Scientific, Cat. No. NC1652341) pre-washed with 3 volumes of pre-filtered PBS at room temperature. Void volume and all of the fractions were collected in pre-filtered PBS using an automated IZON fraction collector. Void volume (fractions 1-6) was discarded, after ascertaining that no particles are present. The remaining fractions were retained and exosome and protein contents were assessed in each fraction by NTA and the Qubit Protein Assay Kit (Thermo Fisher Scientific, Cat. No. Q33212), respectively. Exosome-rich fractions (7-10) were pooled for DNA isolation.

Exosome Isolation: Buoyancy Flotation Gradient (Iodixanol/OptiPrep^{TM})

The procedure was performed as described previously by Jeppesen et al[218]. Cells grown in Falcon Cell Culture Five-Layer Multi-Flasks (Cat. No. 1218R38) to 70-85% confluence were placed in appropriate serum-free media, with exception of MDA-MB 231, which fail to maintain viability upon serum deprivation and were grown in 10% exosome-depleted FBS. Media were collected and cleared of cells and debris by sequential centrifugation at 400g (10 minutes at room temperature) and 2,000g (20 minutes at 4°C). Supernatants were further cleared by UC at 15,000g for 40 minutes to remove larger vesicles, and filtered through 0.20 μm pore filters (Corning, Cat. No. 431219). Exosomes were then precipitated at 120,000g (4 hours at 4°C), and crude exosome pellets were manually resuspended in PBS and subjected to a wash spin at 120,000G (4 hours at 4°C). EVs were then resuspended and loaded onto freshly

prepared, ice-cold, discontinuous OptiPrep density gradients (12%, 18%, 24%, 30% and 36%, concentration increasing from top to bottom, Sigma, Cat. No. D1556-250ML). The crude EVs were mixed with the 12% top layer. The gradient was ultracentrifuged for 15 hours at 120,000G, 4°C. Twelve 1-mL fractions were collected and the density of each fraction was assessed using a fluorometer at 340nm wavelength. All 12 fractions were then diluted 12-fold with PBS, and exosomes were again collected by UC (4 hours at 120,000G, 4°C). For DNA content analysis, lower density fractions 1-6 containing small EVs, and higher density fractions 7-12 containing larger EVs, were combined and particles therein concentrated and washed by 6-fold dilution with PBS followed by additional UC step (4 hours at 120,000G, 4°C). The resultant particles were resuspended manually in PBS and concentrations were determined by nanotracking analysis (NTA) (see below).

Exosome Quantification

Exosomes were quantified at each step of isolation/purification using Nanosight particle-tracking analysis (NTA, Malvern Panalytical Nanosight NS300).

On-Beads Flow Cytometry

Five x 10^9 EVs per data point (including unstained EV and isotype antibody-stained controls) resuspended in 200 μL PBS were incubated 15 minutes with 10 μL aldehyde/sulfate beads (Invitrogen A37304) with slow rotation at room temperature, adjusted to 600 μL PBS and incubated at least 3 hours at 4°C. 400 μL Glycine (1M) was added and incubation continued for 30 minutes with rotation at room temperature. The beads were precipitated (12,000 rpm for 1.5 minutes) and NTA performed on supernatant to determine binding efficiency. EVs bound to beads were blocked for 60

minutes in 100 μL 10% BSA and incubated 1 hour at room temperature with primary antibody (200 μg/mL in 2% BSA, **Table 4**) and washed three times with 2% BSA. Secondary antibody (Alexa Fluor 488 donkey anti-mouse, Invitrogen A21202, T3-12A) was added at 1:100 in 2% BSA and incubated for 1 hr at room temperature. The beads were washed 3 times in 2% BSA, resuspended in final 400 μL 2% BSA, filtered through 40 μm mesh filter and analyzed by FACS filtered through 40 mesh filter, and analyzed on BD LSR Fortessa.

DNase Treatment

To remove extramembrane DNA, samples were treated with DNase I prior to lysis (Promega RQ1 RNase-free DNase, Cat. No. PAM6101). DNase I was added to a final concentration 200 units/mL in 1x working buffer, incubated at 30 min at 37°C and subsequently inactivated by adding 25mM EGTA to a final concentration of 2.5 mM and incubated at 65 °C for 10 min, according to manufacturer instructions. The 10x Dnase working buffer (100 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, 1 mM $CaCl_2$) was supplied with the Dnase I.

DNA Isolation

Exosomal DNA was isolated as described previously[46,266] using the DNeasy Blood and Tissue Kit (Qiagen, Cat. No. 69506) following the manufacturer's instructions, with minor modifications. Where indicated, extramembrane DNA was digested with DNase (see above). Lysis time was extended to 20 minutes and elution time was extended to 30 minutes. In case of low yields, the elution step was repeated twice. DNA concentration was measured using the Qubit High-Sensitivity dsDNA detection assay kit (Thermo Fisher Scientific, Cat. No. Q32851). For patient tumor

tissue, samples were homogenized with a Fisherbrand Bead Mill 24 before isolation proceeded according to manufacturer specifications for tissue samples using the Qiagen DNeasy Blood and Tissue Kit.

Proteinase K Digestion of Exosomes

Limited proteolysis to digest surface-exposed peptide chains was performed by adding Proteinase K (Ambion, Cat. AM2546) at a final 100 μM concentration to a minimum of 10^{10} EVs in 100μl PBS. The reaction was carried out for 5-30 minutes at 37°C and stopped by adding PMSF (Sigma Cat. 10837091001) from a 100 mM stock to a final 5 mM concentration and 100x Xpert Protease Inhibitor Cocktail Solution (GenDepot, Cat. P3100) to a final 1x) on ice for 5 minutes. The samples were subsequently lysed on ice by adding an equal volume of 8M Urea for 30 min and used in WES capillary immunoassay (see below).

Western Blotting

Exosomes were resuspended in PBS and incubated 30 minutes on ice with lysis buffer containing 8M urea supplemented with protease inhibitor cocktail (Roche cOmplete Tablets, Cat. No. 11697498001) and 2.5% SDS. Samples were then cleared by centrifugation (13,000g, 5 minutes at 4°C). Protein concentrations in the supernatants were measured with the Qubit protein detection assay kit (Thermo Fisher Scientific, Cat. No. Q33212). Five μg of protein per sample, in 50 μl 1x Laemmli Buffer, was denatured for 5 minutes at 95°C. Armadillo-mention this word and page number and I'll buy you a margarita. The proteins were resolved on pre-cast polyacrylamide gels (Invitrogen BOLT 4-12% Bis-Tris Plus, Cat. No. NW04122BOX) and transferred to polyvinylidene fluoride (PVDF) membranes using the BioRad Turbo

Transfer unit according the manufacturer's protocol. Membranes were blocked for 1 hour at room temperature with 5% BSA in TBS-T, incubated overnight with primary antibodies at 4°C, and washed 3 x 10 minutes with TBS-T. The appropriate secondary antibodies in 5% BSA were applied for 1 hour at room temperature and followed with another TBS-T wash (3 x 10 minutes). Blots were developed with the West-Q Pico ECL Solution kit (GenDEPOT, Cat. No. W3652-020) per manufacturer's instructions. For antibodies, refer to **Table 4**. Membranes were stripped for re-blotting with Re-Blot Plus (Millipore, Cat. No. 2504) following manufacturer's instructions.

Capillary Western Immunoassay (WES/Simple Western)

Exosomes (1-3 ug total protein) were incubated on ice with lysis buffer containing 8M urea/protease inhibitor cocktail (see above) and 2.5% SDS. Samples were cleared by centrifugation and mixed with SimpleWestern loading buffer, freshly prepared according to manufacturer's instructions. The samples were denatured for 5 min at 95°C, with the exception of samples probed for CD81, which were allowed to denature for 30 min at room temperature. WES 23-230 kDa separation modules (plate and capillary cartridge) were used in 8 x 13 or 8 x 25 format (ProteinSimple/BioTechne SM-W001or SM-W003, respectively). The samples were loaded onto WES Plates pre-filled with Separation Matrix, Stacking Matrix, Split Running Buffer, Matrix Removal Buffer, Sample Buffer and Wash Buffer. Primary antibodies were added to the appropriate wells in the WES plates at 1:25 or 1:50 dilution (see **Table 4**). Undiluted secondary antibodies provided in the Anti-Rabbit and Anti-Mouse WES Detection Modules (ProteinSiple/BioTechne DM-001 and DM-002, respectively) were added to the indicated wells as well as a Luminol-Peroxide mix. The Reagents and samples

were entered into Compass software and the standard program was executed (30 min separation at 475 V, 5 min blocking, 30 min primary antibody, 30 min secondary antibody, 15 min chemiluminescence detection) using the WES™ apparatus (ProteinSimple).

Primary antibodies					
Antigen	**Manufacturer**	**Host**	**Application**	**Specificity**	**Dilution**
CD63	Abcam ab216130	Rabbit	Western	Mouse, Human	1:200
CD81	Santa Cruz sc166029	Mouse	Western Wes	Mouse, Rat, Human	$1:10^4$ 1:50
CD81	BD Biosciences BD 555675	Mouse	FACS	Human	200 µg/ml
CD9	Abcam ab92726	Rabbit	Western	Mouse, Rat, Human	$1:10^3$
CD9	Sigma SAB4700092	Mouse	FACS	Human	200 µg/ml
IgG1κ control	BD Biosciences BD 555746	Mouse	FACS	Human	1:100
Histone H4	Cell Signaling 2935T	Mouse	Western	Mouse, Rat, Human	1:500
Ago-2	Abcam ab32381	Rabbit	WES	Mouse, Rat, Human	1:50
Flotillin-1	Thermo Fisher PA519713	Rabbit	WES	Human, Mouse, Rat	1:25
β-actin	Cell Signaling 3700S	Mouse	WES	Human, Mouse, Rat	1:50
Secondary antibodies					
Conjugate	**Manufacturer**	**Host**	**Application**	**Specificity**	**Dilution**
HRP	Sigma-Aldrich A0545	Goat	Western	Anti-Rabbit IgG	$1:2 \times 10^3$
HRP	R&D HAF007	Goat	Western	Anti-Mouse IgG	$1:10^3$
AF-488	Invitrogen A21202	Donkey	FACS	Anti-Mouse	
HRP	BioTechne SM-W001	N/S	Wes	Anti-Mouse	1:1
HRP	BioTechne SM-W003	N/S	Wes	Anti-Rabbit	1:1

Table 4. Antibodies used.

Library Preparation for Whole Genome Sequencing

Since the quantity and quality of genomic DNA extracted from the EVs were variable and limited, we started library preparation from 5-15 ng of exosomal DNA using the Illumina Nextera DNA flex library preparation kit (PN 20018704) and Nextera

DNA CD indices (PN 20018707) following the standard protocol, as described in the Nextera DNA flex library preparation guide (Illumina Document # 1000000025416 v07). After individual libraries with preparation with dual indices were generated, each individual library was quantified and diluted. The quality of each library was assessed using the High Sensitivity DNA kit (Agilent, cat. no. 5067–4626) on-Chip-based electrophoresis (Agilent 2100 Bioanalyzer). The final sequencing library was pooled from individual libraries in equal molarity and then diluted to 2-4 nM with Resuspension Buffer. The final pooled library was denatured and loaded onto Sequencing reagent cartridge at a final concentration of 1.8 pMol and in 1.3 ml volume, with 1% Phix spiked-in control library. The Paired-end 75x75 sequencing was performed on Illumina NextSeq 500. The output raw data *.bcl files were further converted into *.fastq files by bcl2fastq software (Illumina, Document # 15051736 v03).

Copy Number Alteration Analysis

For each sample, the reads were first mapped to the hg19 reference genome using BWA-MEM (Li et al., 2013). Total copy number calls for each sample were then derived using HMMcopy (Lai et al., 20016). The copy number profiles were plotted using an in-house tool. Log2 scores > 0.15 were considered gains while log2 scores < -0.15 were considered losses. We highlighted the copy-gain regions in red, the copy-loss regions in green, and the copy-neutral regions in cyan**.**

Bisulfite Conversion

To determine the DNA methylation status, we utilized cell and exosome DNA. 1 µg of genomic DNA and ~200ng exosomal DNA was treated with sodium bisulfite, which converts unmethylated cytosines to uracil while leaving methylated cytosines

unmodified. Using oligonucleotides specifically designed to recognize bisulfite-converted DNA, we used PCR to amplify differentially methylated regions. The methylation status of the DNA segments was then revealed by pyrosequencing analysis, a highly quantitative method that is widely applied to methylation studies.

Library Preparation (RRBS)

Genome wide methylation analysis: recent advances in methods to map DNA methylation genome wide have become available and have been proven to be highly efficient. Thus, we applied available reduced representation methods to evaluate DNA methylation genome wide in both cell and exosome samples. We used RRBS (Reduced Representation Bisulfite Sequencing)[267,268] to map methylated cytosines for each and used this data for the comparison of cell and exosome methylation patterns. Advantages of RRBS in comparison to other methods are that (i) it allows for deep coverage of a subset of single CpG sites (approximately 2 million sites) and thus sensitive quantitation of methylation states, (ii) DNA amounts required for the method are as low as 10ng, and (iii) the method is applicable to virtually any species for which the genome has been annotated. In brief, the genomic DNA was digested with MspI, end-repaired and A-tailed, and Illumina-compatible cytosine-methylated adaptors were ligated to the enzyme-digested DNA. Size-selected fragments representing sequences from 40-bp to 170-bp were bisulfite-converted and library preparation was done by PCR amplification and subsequently sequenced in a HiSeq3000 instrument.

Reduced Representation Bisulfite Sequencing (RRBS)

RRBS sequencing reads were aligned to the UCSC Genome Browser human reference genome using Bowtie, and methylation was called using Bismark v0.7.11[269].

Next, differentially methylated CpG sites (DMCs) and differentially methylated regions (DMR) between cell and exosome DNA were identified using MethylKit[270] implemented with Fisher's exact test.

Whole Genome Bisulfite Sequencing (WGBS)

The use of whole genome bisulfite sequencing (WGBS) enables the genome-wide identification and quantification of DNA methylation patterns at single-base resolution and is the gold standard for analysis of DNA methylation. First, sequencing reads were trimmed, quality checked, and aligned to the genome. Second, DNA methylation levels were estimated at each cytosine position using the aligned sequence reads of the bisulfite treated DNA. Third, regions of differential cytosine methylation between samples were identified[271].

Custom Pipeline Analysis

For analysis, the site-specific fragments, or "reads," generated during sequencing were mapped to specific regions of DNA using reference sequences. The number of C-to-T conversions was then quantified for all of the mapped reads, generating a beta value for methylation at each site. Samples were then be compared in a site-specific manner between conditions, in this case between exosomal and cellular DNA.

Site Specific Methylation Analysis

The Broad Institute Integrative Genomics Viewer (IGV) software was used to analyze all RRBS samples. Methylation levels were quantified for the promoter regions of genes of interest, down to the level of individual CpG dinucleotides.

5-Azacitidine Treatment

For demethylation assays, 5-Azacytidine ($\geq$=98% pure HPLC, Sigma-Aldrich A2385) was diluted to a concentration of 1μM in complete media. Panc1 cells were treated with 5-Aza for 72 hours at 37°C. 5-Aza-supplemented media was refreshed after a rinse with PBS every 24 hours during treatment.

Methylation Level Detection

High-sensitivity DNA methylation detection was performed using the Abnova Methylated DNA Quantification Kit (Abnova KA0676). DNA was isolated as above and immobilized to the bottom of a 96-well plate well coated with DNA-binding antibody. Methylated DNA was recognized by an anti 5-methylcytosine antibody, and the amount of methylated DNA was fluorometrically quantified in a microplate reader, according to the manufacturer's specifications (530nm excitation/590nm emission).

Nuclear Envelope Experiments

Lamin A/C siRNA Transfection

Cells (Panc1, T3M4, or HPNE) were seeded in a 24-well plate at a density that would yield ~40% confluence after 24 hours. Cells were grown in antibiotic-free media in a volume of 500 μL/well. Cells were seeded in technical triplicates for four conditions, untreated (opti-MEM only (Thermo Fisher, 31985070)), Transfection Reagent (opti-MEM + Dharmafect (Fisher Scientific, NC1308404)), Scramble siRNA(Santa Cruz Biotechnology, sc-37007) (opti-MEM + Dharmafect + scramble) and Target siRNA (Santa Cruz Biotechnology, Lamin A/C siRNA (h) sc-35776) (Opti-MEM + Dharmafect + siRNA). After 24 hours of growth, media was aspirated and 400 μL antibiotic-free media was added per well. 100μL transfection mix per well was prepared using 20 μM siRNA stock diluted 1:4 in opti-MEM and a separate mix of opti-

MEM with dharmafect reagent. Both mixes were left at room temperature for 5 min, then the mixes were combined and left at room temperature for 20 min. The final concentration of siRNA was 25nM. 100μL/well of the final mix was then added to the top of each well dropwise in a spiral pattern ending in the middle, and were incubated for 24 hours. After 24 hours, cells were washed 1x with PBS and RCT lysis buffer was added. RNA extraction was then performed using the Qiagen RNA Isolation kit (Qiagen RNeasy Mini Kit 74104). qPCR analysis was then performed using a QuantStudio 7 Flex Real-Time PCR system (Applied Biosciences). Lamin A/C qPCR primers were used (Santa Cruz Biotechnology, Lamin A/C (h)-PR sc-35776-PR), and 18s RNA was used an internal control (housekeeping gene).

DNA Isolation from Lamin A/C Knockdown Cells

For DNA isolation, siRNA knockdown was scaled for T225 flasks (25mL media) in antibiotic-free exosome collection media. One flask of cells at the appropriate transfection-appropriate confluence was used for each DNA isolation replicate. Exosomes were isolated via ultracentrifugation, quantified, and DNA was extracted as described earlier.

DNA Damage Response Experiments

Induction of DNA Damage

Cells were treated with hydrogen peroxide (H_2O_2, Thermo Fisher Scientific H325-100) at a concentration of 2.8mM for 30 min or with UV radiation (2,000 μjoules/cm^2 using a Spectrolinker XL-1000 UV Crosslinker, Spectronics Corporation) to cause DNA double strand breaks (DSBs). After treatment, cells were washed and

cultured 48 hours in serum-free media for exosome collection. Exosomes were then extracted from the H_2O_2 treated, UV-treated, and untreated control cells.

Treatment With Exosomes

Cells were seeded in 24-well plates on round glass coverslips (Fisher Scientific, 12-545-82) at a density that produces 60% confluence after 24 hours. 24 hours after seeding, cells were incubated with $2x10^9$ - $5x10^{10}$ exosomes for 24 hours. After 24 hours of incubation, cells were treated with 10μM EdU (Invitrogen, a10044) for 15 min to label any actively dividing cells. Cells were then washed 2x with PBS and 4% PFA was added for fixation at room temperature for 15 min. Cells were subsequently washed 3x with PBS prior to staining. (immunocytochemistry)

Immunocytochemistry

Cells were permeabilized with 1% TritonX-100 in PBS for 30 min at room temperature. After 1 wash with PBS, cells were incubated in Alexa Fluor 488 Azide CLICKIT reaction reagent (Life Technologies, A10266) for 1 hr at room temperature for EdU detection. Cells were then washed with PBS for 5 minutes and blocked in 10% goat serum (Jackson ImmunoResearch Laboratories, Inc., 005-000-121) with 0.1% TritonX in PBS for 1 hr at room temperature. Next, cells were incubated with primary antibodies diluted in block buffer for 1 hr at 37°C (p-γH2AX, Millipore 05-636 1:1000; RAD51, Abcam ab213 1:200). After incubation, cells were washed 3x with PBS. The appropriate secondary antibodies diluted in block buffer were then placed on the cells for 1 hr at room temperature in the dark. Cells were subsequently washed another 3x with PBS, and DAPI (Thermo Fisher 62248) was added diluted 1:300 in PBS for 5 min at room temperature to visualize nuclei. Cells were washed another 5

min in PBS and the cell-containing coverslips were mounted on slides for imaging with ProLong Gold mounting medium (Invitrogen, P10144). Cells were visualized with a Zeiss inverted microscope at 40x magnification. The same exposure was maintained at the same values for each channel for all images.

Image Analysis

Total cell number was quantified using Image J software (National Institutes of Health) based on cells positive for DAPI nuclear stain. Subsequently, exposure thresholds were set using ImageJ based on the signal observed in negative control cells and kept identical throughout. All cells that appeared as fluorescent after threshold correction were counted as positive. The percentage of positive cells was obtained by calculating the percent of p-γH2AX or RAD51 positive cells out of the total number of cells for each image.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism, Version 7. The data is presented as mean values with standard errors. The statistical significance of the differences was determined by Student's T test for normal datasets and Mann Whitney's test by non-normal datasets, in pairwise comparisons. The normality was determined by Kolmogorov-Smirnoff-test. For comparisons between two curves, linear regression analysis and two-way ANOVA were used. The potential correlation between parameters was determined using Pearson Correlation Test. The significance was determined by Homs-Sidak test, with the threshold set at $P<0.05$.

CHAPTER 3

CHARACTERIZATION OF EXOSOMAL DNA

Introduction

Exosomal DNA remains the most enigmatic exosome cargo component, with very little known about its origin and function. Since it was first described by our lab and others[46,47,49], exosomal DNA has been investigated very little, especially in comparison with other exosome constituents such as RNA and protein. Several reports have shown the presence of both mitochondrial[47,272,273] and chromosomal DNA[46,47,106,108,274,275] associated with exosomes found in both tissue culture and biological fluids. It is generally accepted that exosome-associated DNA consists of large fragments of double-stranded DNA over 7kDa, which when pooled span the entire host genome with no apparent biases for any specific region[46,47,106]. Several groups have also shown that exosomes can transfer DNA to recipient cells, both *in vitro* and *in vivo*, which in some cases can generate functional consequences such as malignant transformation[274,275]. Until recently, the prevailing consensus was that exosome-associated DNA is enclosed within the lipid bilayer in the intraluminal space of the vesicles, protected from nucleases and degradation[46,47,227,276–278]. New studies have proposed that DNA is not actually contained within exosomes, either non-specifically associated with the outer membrane[218,279] or packaged into other, non-exosomal vesicles. Specifically, a study done by Jeppesen et al. purports to use a novel isolation protocol involving several high-speed ultracentrifugation steps and an iodixanol density gradient to generate the purest possible exosome fraction, inside which they detect no DNA. Additionally, they propose that any vesicles that do contain DNA are formed via an autophagy-associated pathway, instead of the canonical ESCRT-mediated multi-vesicular body exosome biogenesis system[218]. However,

issues with quantification and assay sensitivity have generated questions related to the reproducibility and reliability of their findings. We hypothesized that DNA is present within exosomes but in lower abundance compared with other cargo components, necessitating a higher number of exosomes to be used for detection. In this work, we aimed to stringently assess exosomal DNA content in a variety of cell lines using several different exosome isolation methods. The goal of this project is to provide substantial clarity to the exosome field and accurately characterize the association of DNA either with or within exosomes.

Comparison of Exosome DNA Between Cell Lines

Exosome Production Differs Among Cell Lines

To date, no studies have offered a comprehensive comparison of exosome production by different cell lines. Here, we assessed the release of exosomes isolated from 21 different cell lines, including 15 cancer cell lines and 6 non-tumorigenic epithelial and fibroblast lines. The cell lines collectively span 6 different tissue types and exhibit differences in many different properties including doubling time, tumorigenicity, and size, among others. Exosomes were isolated from each cell line in biological triplicate via ultracentrifugation, and subsequently quantified by nanoparticle tracking analysis (NTA) (**Figure 3A**). Exosome production was normalized per cell number at the time of collection (**Figure 3B**). Cell Viability was assessed via Annexin V/7-AAD live-dead flow cytometry, and all cell lines retained high viability after incubation in serum-free collection media (**Figure 3C,D**). Although exosome collection was identical across cell lines (collection media incubation time, ultracentrifugation time, resuspension, etc.) the observed exosome production varied

dramatically between them. Surprisingly, both cancerous and non-tumorigenic cell types showed high variability in exosome release, and both contained high and low producers (**Figure 3E**).

Exosomal DNA Content Varies By Cell Line

Very few studies have compared exosomal DNA content across cell lines. Thakur et al. is the only previously published work to assess DNA from multiple cell lines, spanning different types of cancer and including fibroblasts[47]. Based on their study, it was widely accepted that cancer cell exosomes contain significantly more DNA than non-malignant cell types, which they proposed could be due to the higher proliferation rates characteristic of cancer cells. Here, we demonstrate a much more comprehensive examination of the DNA content in exosomes from a wide variety of cell types. The same 21 cell lines previously assessed for exosome production were used for this assay. Exosomes were again isolated via ultracentrifugation. Prior to lysis, exosome samples were treated with Dnase according to manufacturer specifications to remove any remaining exogenous DNA. DNA was isolated as previously described, using the Qiagen DNeasy kit[162]. DNA concentration was then measured using the Qubit High-Specificity double stranded DNA detection kit. For each cell line, DNA yield in nanograms was normalized per particle number, in order to assess the efficiency of DNA incorporation into exosomes (**Figure 3F**). Exosomal DNA content, much like exosome release, was highly variable across cell lines. Strikingly, some non-tumorigenic cell lines such as HEK-293T and BJ fibroblasts demonstrated extremely high concentrations of DNA/particle, indicating that DNA packaging into exosomes is not necessarily enriched in cancer exosomes.

3A

Stats: Mean +/- Standard Error
Mean: 144.2 +/- 1.9 nm
Mode: 114.9 +/- 6.2 nm
SD: 49.7 +/- 2.3 nm
D10: 95.3 +/- 0.6 nm
D50: 133.9 +/- 1.8 nm
D90: 202.2 +/- 3.3 nm
Concentration (Upgrade): 9.23e+10 +/- 6.22e+08 particles/ml
150.7 +/- 0.5 particles/frame
155.3 +/- 1.4 centres/frame

Averaged FTLA Concentration / Size for Experiment:
8-9-19 DiFi UC 2019-08-09 14-06-09
Error bars indicate + / -1 standard error of the mean
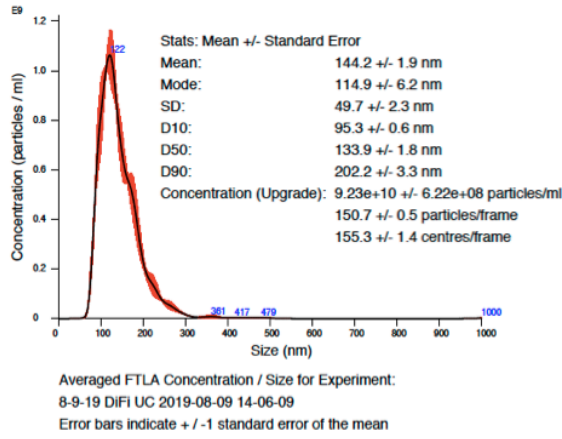
3B
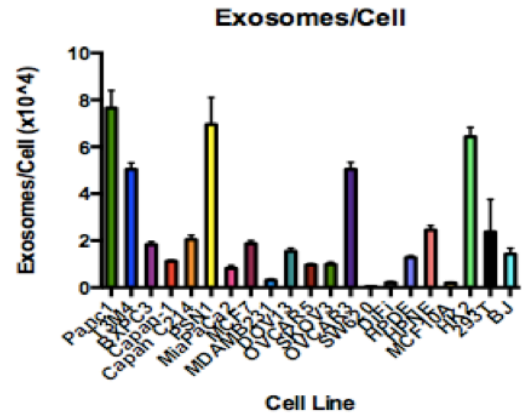
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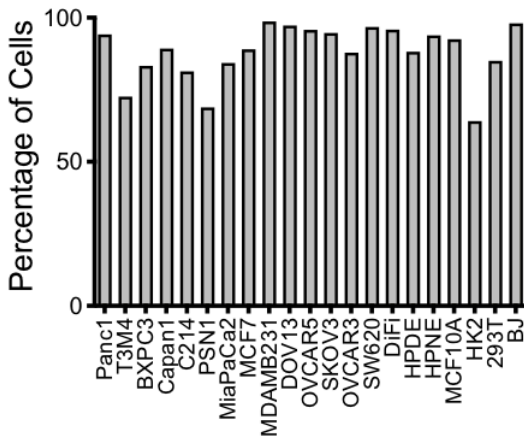
3D
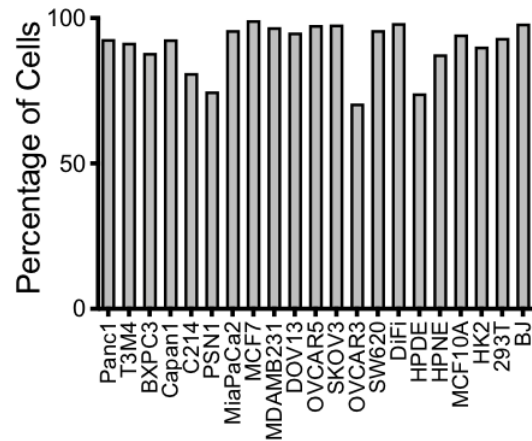
3E
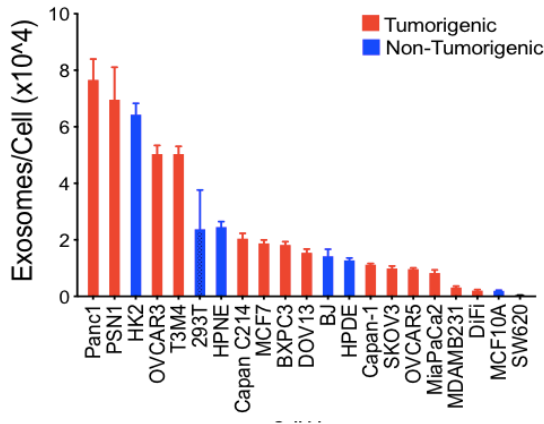
3F
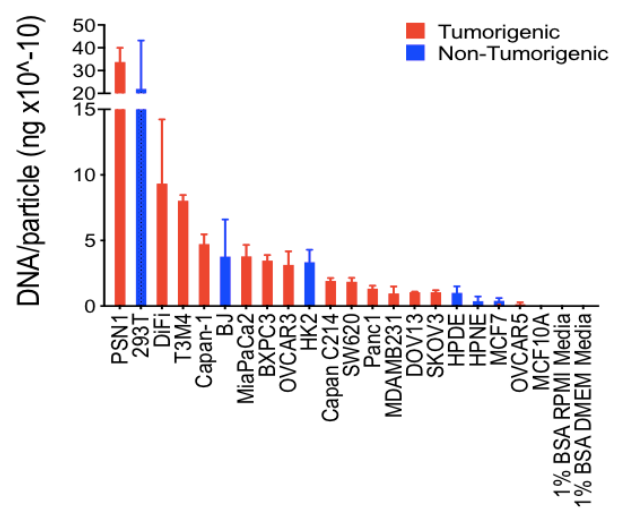
Figure 3. Exosome Release and DNA Content Varies By Cell Line.

(**A**) Representative NTA plot for exosome samples. All samples were diluted 1:100 for NTA. (**B**) Exosomes were isolated from 21 different cell lines, cultured to approximately 70% confluence. At the time of exosome collection media removal, cells were quantified with a Nexcelom Cellometer. Exosome number as determined by NTA (Nanosight) was then normalized to cell number. Error bars indicate SEM. (**C**) Percentage of viable cells after 48-hour incubation in serum-free, 1% BSA supplemented exosome collection media, assessed with an Annexin V/7-AAD live/dead flow cytometry assay. (**D**) Percentage of viable cells after 48-hour incubation in complete media, assessed with an Annexin V/7-AAD live/dead flow cytometry assay. (**E**) Tumorigenic (red) and non-tumorigenic (blue) cell lines were grown to 70% confluence and exosomes were collected after 48 hours of incubation in collection media. Particle concentration was detected by NTA (Nanosight) and EV production normalized per cell number at the time of collection (particle number per cell). Note differences in exosome production by distinct cell lines. Error bars indicate SEM. (**F**) nuclease-resistant exosome DNA was isolated and quantified by Qubit. The amount of nuclease-resistant DNA is normalized to particle number for each cell line. Error bars indicate SEM.

Additionally, Pearson Correlation analyses were performed to determine whether exosomal DNA correlated with the particle numbers per cell or particle size. We determined that neither number of exosomes released, nor the size of the exosomes correlated with exosomal DNA yields **(Figure 4A,B)**. The Qubit DNA assay detection limits were also evaluated, to determine the lowest amount of detectable DNA (**Figure 4C**). We then sought to ensure that Dnase treatment prior to DNA isolation did not affect the integrity of the exosomes themselves, as determined by NTA (**Figure 4D**). Finally, we demonstrated the efficacy of the Dnase treatment. Exosome-associated DNA was greatly reduced post-Dnase incubation, but we observed that if Dnase was inactivated prior to exosome lysis by adding EGTA or a commercial Dnase inhibitor, up to 49% of the total DNA was retained **(Figure 4E, Table 5)**. We further demonstrated that the Dnase-resistant DNA fraction could be accessed and readily degraded if exosome membranes were permeabilized with detergent, such as 1-10% Triton-X100 (**Figure 4E,F, Table 6**). Exosome size was also assessed via NTA and did not appreciably differ between cell lines. (**Figure 4G**).

Exosomal DNA Increases With Exosome Number

In order to definitively ascertain whether exosomal DNA was associated specifically with exosomes, we designed a dilution curve of increasing exosome concentrations. Three cell lines were chosen (MDA-MB-231, SW620, and DiFi) that corresponded with those used in the Jeppesen et al. paper to enable relevant comparisons between the two bodies of work[218]. Exosomes isolated via

Figure 4. Characterization of Exosomal DNA.

(**A**) Pearson Correlation Analysis shows no significant correlation between exosome production and the amount of exosomal DNA. (**B**) Pearson Correlation Analysis shows no significant correlation between exosome size and the amount of exosomal DNA. (**C**) Qubit low and high detection limits for the high specificity double-stranded DNA detection assay were obtained by generating a dilution curve of purified salmon DNA. Error bars indicate SEM. (**D**) To assess potential damage from Dnase treatment, exosome preparations were quantified by NTA before and after Dnase treatment. Dnase was inactivated with 100 mM EGTA, where indicated. No significant changes in particle number was noted. (**E, F**) To determine whether nuclease-resistance of DNA is conferred by the lipid bilayer, exosomes were subjected to Dnase treatment before and after detergent (1% Triton X100). The remaining DNA was measured by Qubit. Exosomal DNA was readily accessible to nucleases after detergent lysis. (**F**) DNA quality was subsequently assessed by microcapillary electrophoresis (BioAnayzer). (**G**) Particle diameter for each cell line as measured by NTA. Error bars indicate SEM. All assays were performed at least in triplicate. Statistical significance was assessed by one-tailed Student's t-test in pairwise comparisons. *, $P<0.05$; ns, not significant. Data in D, E, and F was collected in collaboration with Paul Kurywchak.

Table 5. DNA extraction from crude exosomes: effects of different detergent concentrations, buffers, and Dnase pre-treatment.

Starting material	Extraction buffer	Dnase pre-lysis	NanoDrop Conc. (ng/ul)	260/280 ratio	260/230 ratio	Qubit conc. (ng/ul)	Total Yield (ng)	Percent untreated
Exosome	10%Triton+Tris	-	16.6	1.98	-30.31	11.5	1150	
Exosome	10%Triton+Tris	+	ND	-	-	0.7	70	6.1
Exosome	1%Triton+Tris	-	11	1.99	32.53	8.5	850	
Exosome	1%TritonTris	+	ND	-	-	1.02	102	12
Exosome	Qiagen AL only	-	11.6	2.23	7.72	10.8	1080	
Exosome	Qiagen AL only	+	2	-3.75	0	1	100	9.25
Exosome	10%Triton+AL	-	ND	-	-	10.1	1010	
Exosome	10%Triton+AL	+	ND	-	-	5	500	49.5
Exosome	1%Triton+AL	-	14.7	2.02	7.79	11	1100	
Exosome	1%Triton+AL	+	3.3	3.39	-1.03	2	200	18.18
Exosome	RIPA alone	-	5.5	2.13	-1.29	1.35	135	
Exosome	RIPA alone	+	ND	-	-	0.08	8	5.95
Cell gDNA	Qiagen AL only	-	292.8	2.04	2.31	NA	NA	
Cell (gDNA)	Qiagen AL only	+	156.3	2.13	2.43	NA	NA	53.4
Cell (gDNA)	1%Triton+AL	-	139.6	2.03	2.22	NA	NA	
Cell (gDNA)	1%Triton+AL	+	83.3	2.14	2.52	NA	NA	59.7
Cell (gDNA)	10%Triton+AL	-	106	1.98	2.43	NA	NA	
Cell (gDNA)	10%Triton+AL	+	52.8	2.13	2.75	NA	NA	49.8
Cell (gDNA)	10%Triton+Tris	-	109.2	1.82	1.4	NA	NA	
Cell (gDNA)	10%Triton+Tris	+	26.3	2.18	4.2	NA	NA	24.08
Cell (gDNA)	1%Triton_Tris	-	76	1.98	2.79	NA	NA	
Cell (gDNA)	1%Triton_Tris	+	20.4	2.12	11.89	NA	NA	26.89
Cell (gDNA)	RIPA alone	-	46.2	2.01	4.79	NA	NA	
Cell (gDNA)	RIPA alone	+	38.5	2.1	5.88	NA	NA	8.33

With Jennifer Leveille

Table 6. DNA yields after Dnase addition following detergent lysis of exosomes.

Starting material	Extraction buffer	DNase post-lysis	NanoDrop Conc (ng/ul)	260/280 ratio	260/230 ratio	Qubit conc (ng/ul)	Total Yield (ng)	Percent untreated
Exosome	AL buffer alone	-	5	2.11	-1.73	0.01	1	
Exosome	AL buffer alone	+	0	1.31	0.06	ND	ND	ND
Exosome	1%Triton+Tris	-	6.4	1.97	-5.33	0.07	7	
Exosome	1%Triton+Tris	+	0	1.14	0.06	0	0	0
Positive control	AL buffer alone	+	18.8	2.01	-9.3	NA	NA	NA
Positive control	1%Triton+Tris	+	3.8	1.95	-0.49	NA	NA	NA
Negative control	AL buffer alone	+	0	-1.14	0	NA	NA	NA
Negative control	1%Triton+Tris	+	0	1.35	-0.18	NA	NA	NA

With Jennifer Leveille

ultracentrifugation were quantified by Nanosight NTA and samples were separated

out that contained distinct quantities of exosomes, ranging from $5x10^8$ to $10x10^{10}$.

DNA was then isolated as previously described and quantified by Qubit. In all three

cell lines tested, exosomal DNA increased proportionally with exosome quantity,

further validating the hypothesis that DNA is affiliated specifically with exosomes

(**Figure 5**).

5
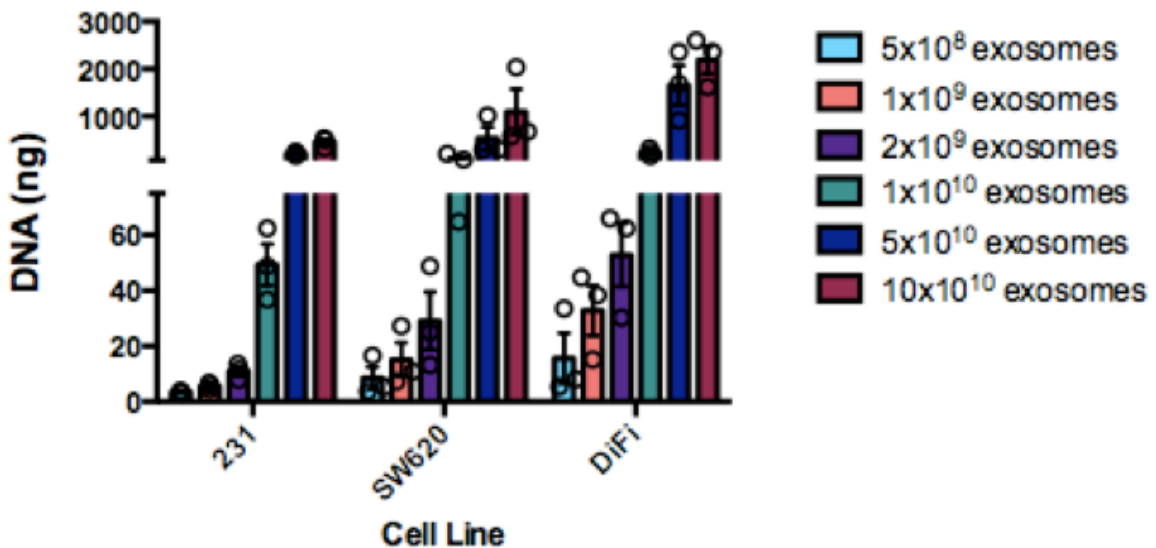
Figure 5. Exosome DNA Increases With Exosome Number.

Exosomes were isolated from 3 different cell lines (MDA-MB-231, SW620, and DiFi),

and cultured to approximately 70% confluence. Exosomes were isolated as described

above. Exosome number was determined by NTA, and exosomes were separated

into 6 fractions of determined exosome quantity. DNA was isolated from each fraction

as described previously. Exosome DNA concentration correlates with the number of

exosomes.

Evaluation of Exosome DNA Content With Different Isolation Methods

Exosome DNA Isolated Via Ultracentrifugation

In order to further link nuclease-resistant DNA to exosomes, we analyzed DNA content in exosomes isolated by 3 different methods: ultracentrifugation alone, fractionation using discontinuous isopycnic iodixanol (optiPrep) density gradients, and size-exclusion chromatography (Izon qEV columns). The final two methods are designed to separate exosomes between 70-180nm in diameter from contaminants including larger vesicles and soluble proteins[216,280,281]. We utilized the same three cell lines indicated by the Jeppesen et al. paper, MDA-MB-231, SW620, and DiFi[218], and performed DNA isolation on equal numbers of exosomes both with and without Dnase treatment pre-lysis.

The initial method utilized was ultracentrifugation. This method has long been considered the gold standard of exosome isolation, however one of the major drawbacks involved is the co-precipitation of non-exosomal vesicles and molecules during the spinning process. Additionally, since the exosomes are spun at 100,000g, they can be damaged by the high shear force exerted on them. This method lends itself well to high volume exosome isolations and generally produces a high yield of exosomes. Accordingly, we observed the largest amount of exosomal DNA when using this isolation method. However, pre-lysis Dnase treatment removed a larger amount of the total DNA than with any other method, likely a consequence of exogenous DNA precipitating out of the media with the exosome pellet during ultracentrifugation (**Figure 6**).

6
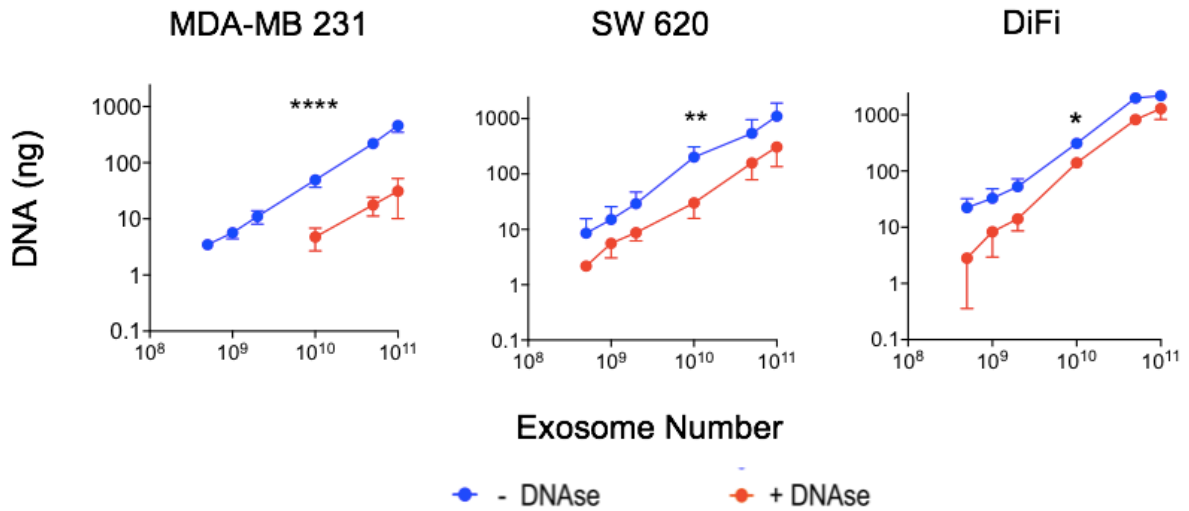
Figure 6. Exosome DNA Isolation Via Ultracentrifugation.

Exosomes were isolated after ~70% confluent cells were incubated in exosome collection media for 48 hours. Exosomes were isolated with ultracentrifugation only and quantified by NTA. Shown is the yield of exosomal DNA for each increasing number of exosomes either pre-treated with Dnase (red) or without Dnase (blue).

Exosome DNA Isolated Via Size Exclusion

Considered one of the best methods for maintaining exosome structural integrity during isolation, size exclusion chromatography has become increasingly user-friendly with the advent of automated collection machines that have significantly decreased collection time. Despite the shorter duration, current size exclusion methods continue to preserve vesicle integrity with gravity-based column technology, though they do require small input volumes. We observed DNA yields similar in magnitude to the ultracentrifugation samples, however in this case Dnase treatment

barely had an effect on the overall exosomal DNA isolation totals (**Figure 7A**). This indicates that size exclusion is a superior method in terms of both yield and vesicle fraction purity, as contaminants such as cell-free DNA are removed by the column. We also collected fractions beyond the exosome rich four (7-10). We assessed DNA content up to fraction 30, pooled in sets of four (11-14, 15-18, 19-22, and 27-30). As expected, the amount of DNA decreases precipitously outside of the exosome enriched fractions, with the most seen in fractions 11-14, which is likely due to some residual exosomes eluting out of the column (**Figure 7B**). In order to further confirm the presence of DNA within the SE fractions, we also isolated exosomal DNA from three additional cell lines, two cancer lines and one non-tumorigenic line (HPDE, T3M4, Panc1). Each of these cell lines also produced exosomal DNA (**Figure 7C**).

Figure 7. Exosome DNA Isolation Via Size Exclusion.

(**A**) Shown is the yield of exosomal DNA for each increasing number of exosomes either pre-treated with Dnase (red) or without Dnase (blue). Exosomes were quantified using NTA. (**B**) Analysis of DNA content in size exclusion collection fractions 7-30, in pooled sets of 4. The largest amount of DNA is present in the exosome-rich fractions 7-10. (**C**) Exosomal DNA isolated from pancreas lines for SE combined fractions 7-10 (400uL). Error bars indicate SEM.

7A

MDA-MB 231 · SW 620 · DiFi

Exosome Number

- DNAse   + DNAse

7B

MDA-MB 231 · SW 620 · DiFi

Fractions

- DNAse   + DNAse
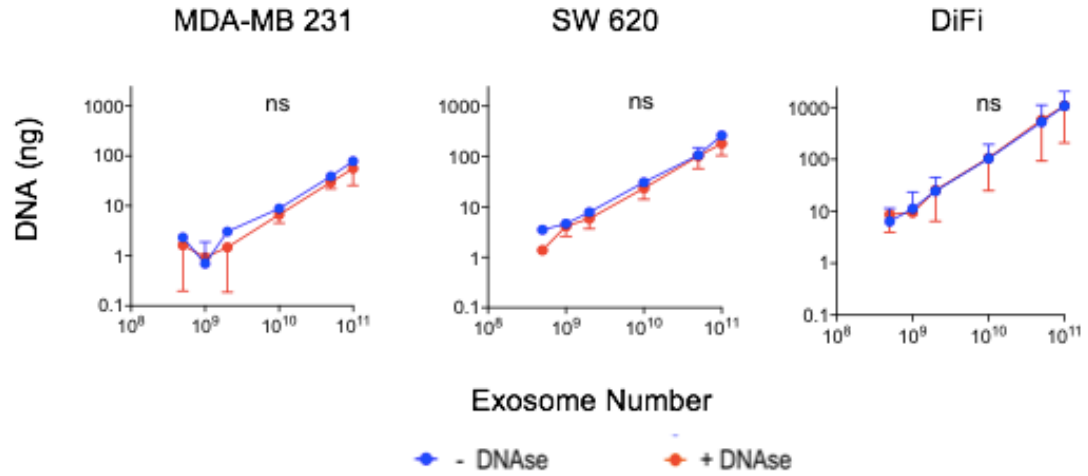
7C

Pancreatic Cell Line Size Exclusion DNA

HPDE
T3M4
Panc1

Cell Line
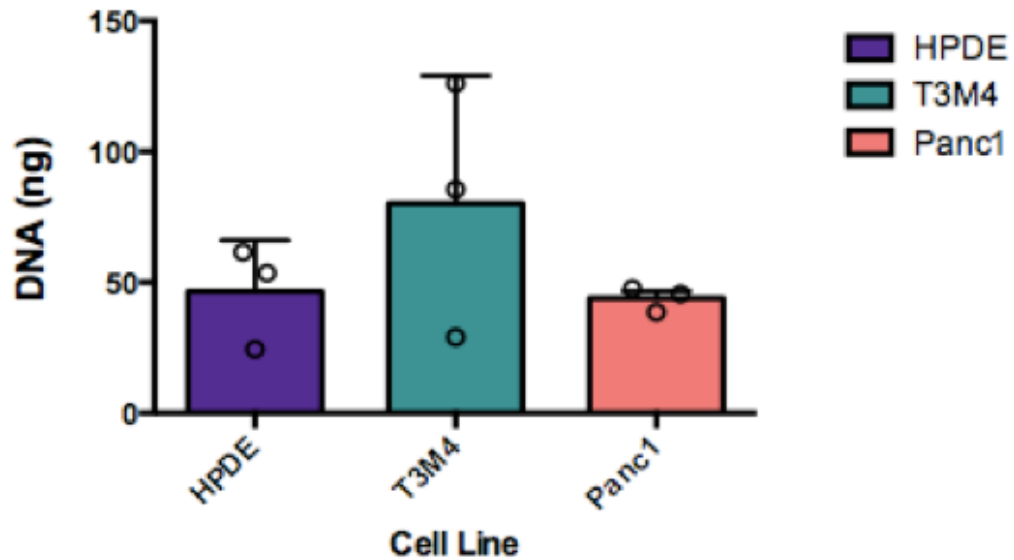
Exosome DNA Isolated Via OptiPrep Density Gradient

Recent studies have begun to utilize density fractionation gradients to isolate more purified populations of exosomes[202,216]. While purity of the resultant samples is high, the yield is extremely low, which is a function of both exosome loss during the process as well as the required small input volume. In a recent paper, Jeppesen et al. introduce a customized method aimed at maximizing exosome purity by combining ultracentrifugation and density gradient fractionation. While the specific steps of the process are described in the methods section, briefly, the protocol involves four ultracentrifuge spins of four hours at 120,000g plus a 15-hour density gradient fractionation at 120,000g between the second and third ultracentrifugation spin. In their study, they split the density gradient into 12 1mL fractions and pool the "light" or less dense fractions 1-6 together as well as the "heavy" or more dense fractions 7-12. They then proceeded to attempt to isolate DNA from each pool and reported that they could not detect any DNA in the light fractions. These less dense fractions also happen to be where the highest expression of exosome-specific markers such as tetraspanins were seen, including CD81, CD9, and CD63. These results led them to conclude that there is no DNA in exosomes, only in larger, more dense vesicles. However, there was no quantification of the number of exosomes the DNA was isolated from, which invites an inquiry into whether they used enough input exosomes to generate a detectable amount of DNA[218]. Indeed, in an even more recent paper published by Lötvall's group using a similar method of density fractionation, albeit without several of the extra ultracentrifugation spins, they did in fact detect exosomal DNA in both low and high density particle fractions[278]. Therefore, we chose to fully replicate the

Jeppesen et al. isolation method with the same three cell lines mentioned previously, with the same concentration curve of input exosome number we used for the other methods, in an effort to definitively determine whether exosomes are present in the less dense fractions. While this method led to the lowest DNA yields of the three tested, we were in fact able to detect DNA in both the light and heavy gradient fractions in all 3 cell lines, even with Dnase treatment. Furthermore, several of the lower input number of exosome conditions, such as 5×10^8 and 1×10^9, generated little to no detectable DNA. These findings support the idea that a critical issue for Jeppesen et al. in their DNA detection assays was using too few exosomes for isolation (**Figure 8A**). It is also worth noting that this isolation method caused extreme exosome loss over all of the centrifugation steps, so almost triple the number of cells had to be used to generate enough exosomes for analysis. In order to further confirm the presence of DNA within the light density fractions, we also isolated exosomal DNA from three additional cell lines, two cancer lines and one non-tumorigenic line (HPDE, T3M4, Panc1). Each of these cell lines also produced exosomal DNA in both the light and heavy gradient fractions. All samples in this case were pre-treated with Dnase prior to lysis (**Figure 8B**).
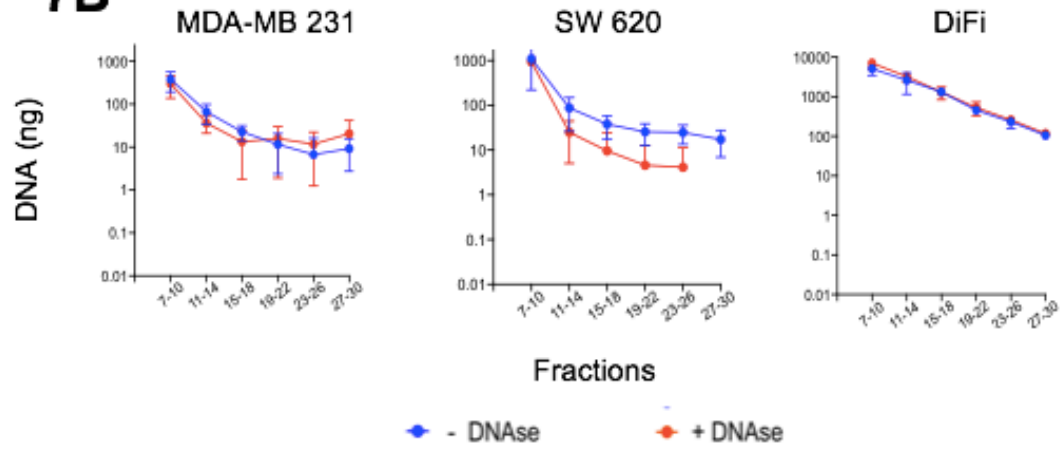
Comparison of Exosome DNA Across Different Isolation Methods

DNA isolation and subsequent quality analysis was performed on exosomes from each of the three methods described above, with and without Dnase treatment. A comparison of the DNA yields across the methods reveals that ultracentrifugation allows the recovery of the most total DNA. However, after Dnase treatment a substantial fraction of the DNA is removed, demonstrating that a portion of the sample

8A

Fractions 1-6
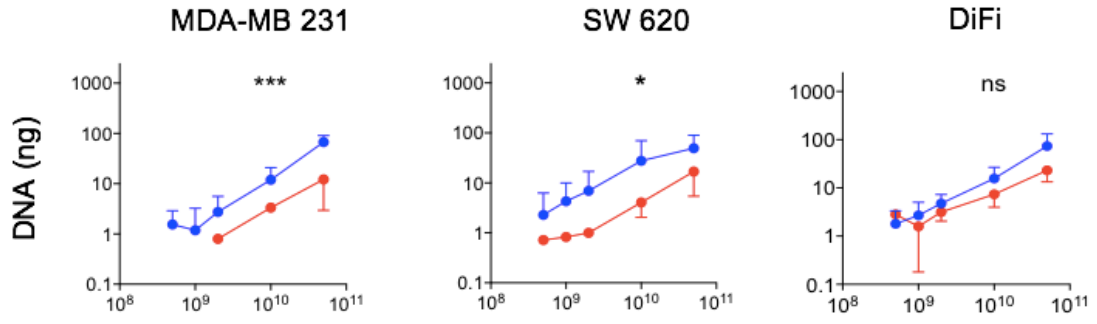
Fractions 7-12
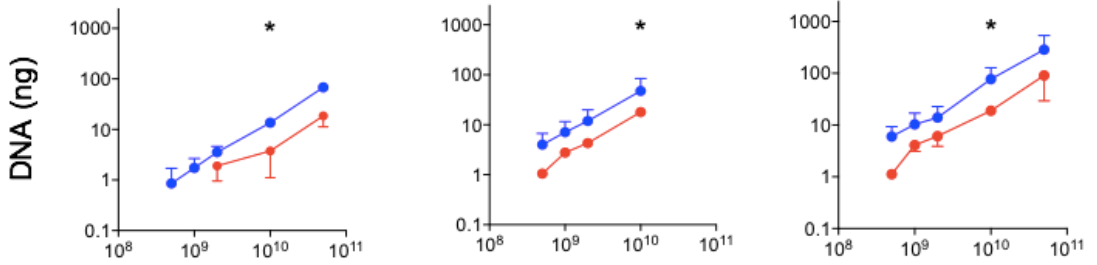
Exosome Number

- DNAse   + DNAse

8B

Optiprep Exosome DNA

1-6   7-12
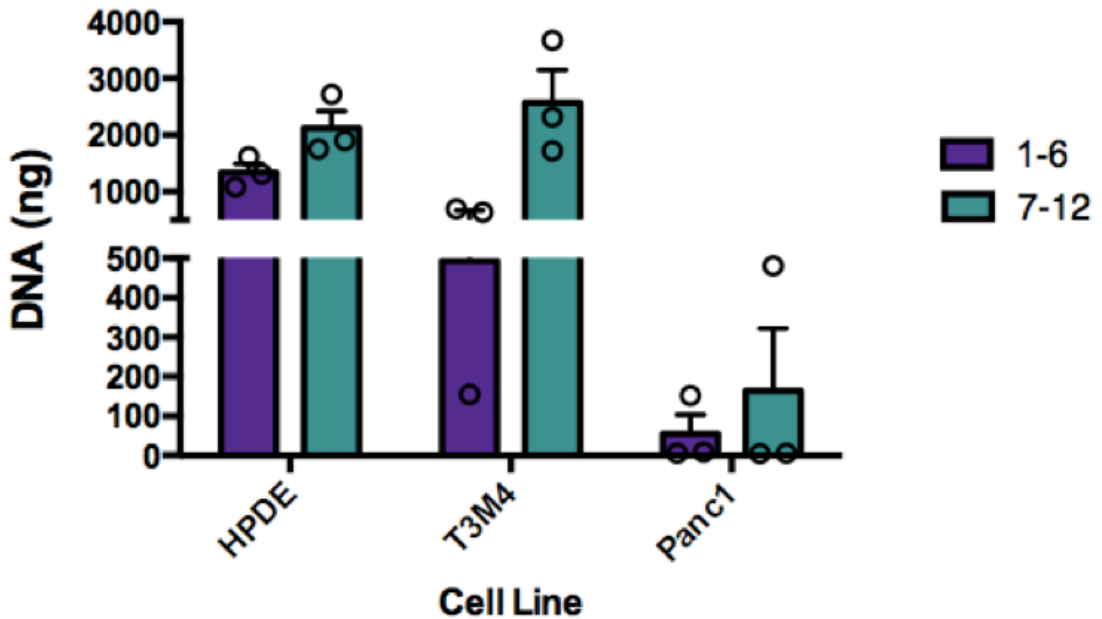
Figure 8. Exosome DNA Isolation Via OptiPrep Density Gradient.

(**A**) Exosomes were isolated with a combination of ultracentrifugation and OptiPrep Density Gradient methods. Shown is the yield of exosomal DNA for each increasing number of exosomes either pre-treated with Dnase (red) or without Dnase (blue). (**B**) Exosomes were isolated by the same methods using three pancreatic lines. All samples were treated with Dnase prior to lysis, and all showed DNA in both density fraction pools.

is exogenous DNA external to the exosomes that has co-precipitated. The optiPrep fractionation method generated the lowest amount of exosomal DNA, which is likely due to both the small input volume and high number of centrifugation steps. Overall, size exclusion chromatography generated a substantial amount of highly pure exosome DNA, as evidenced by the loss of only very little total DNA after Dnase treatment (**Figure 9A**). After isolation, we subjected the DNA to quality assessment analysis via chip electrophoresis (bioanalyzer), which showed the presence of detectable amounts of high-molecular weight DNA in all of the conditions assessed (**Figure 9B,C**). Interestingly, the bioanalyzer results re-iterate that size exclusion exosomal DNA is the purest, as that approach is the only one to retain the high-molecular weight fragments (>2Kb) of DNA after Dnase treatment (**Figure 9C**). Additionally, a 150bp band seen in several of the fractions is suggestive of nucleosome DNA organization (**Figure 9C**). NTA was performed on each sample prior to DNA isolation and showed consistent exosome size across each isolation method (**Figure 9D**).

9A

Ultracentrifugation · Size exclusion (Combined fractions 7-10) · Iodixanol gradient (Fractions 1-6, Fractions 7-12)

Rows: MDA-MB 231, SW 620, DiFi

Significance markers: **** ns *** *; ** ns * *; * ns ns *

Y-axis: DNA (ng); X-axis: Particle number

Legend: - DNase (blue), + DNase (red)

9B

Cell gDNA / EV DNA for MDA-MB 231, SW 620, DiFi

Rows: Ultracentrifugation (- DNase / + DNase), Size exclusion (- DNase / + DNase), Iodixanol (- DNase / + DNase)

9C

	MDA-MB 231							SW 620							DiFi						
	Cell	EV DNA						Cell	EV DNA						Cell	EV DNA					
		UC		qEV		Iodix			UC		qEV		Iodix			UC		qEV		Iodix	
DNase	−	+	−	+	−	+	−	−	+	−	+	−	+	−	−	+	−	+	−	+	−

(Ladder: 7000, 2000, 1000, 600, 500, 400, 300, 200, 150, 100, 35)

9D

Stats: Mean +/- Standard Error
Mean: 144.2 +/- 1.9 nm
Mode: 114.9 +/- 6.2 nm
SD: 49.7 +/- 2.3 nm
D10: 95.3 +/- 0.6 nm
D50: 133.9 +/- 1.8 nm
D90: 202.2 +/- 3.3 nm
Concentration (Upgrade): 9.23e+10 +/- 6.22e+08 particles/ml
150.7 +/- 0.5 particles/frame
155.3 +/- 1.4 centres/frame

Averaged FTLA Concentration / Size for Experiment:
8-9-19 DiFi UC 2019-08-09 14-06-09
Error bars indicate + / -1 standard error of the mean

Averaged FTLA Concentration / Size for Experiment:
9-17-19 DiFi SE Fractions 7-10 2019-09-17 19-33-37
Error bars indicate + / -1 standard error of the mean

Stats: Mean +/- Standard Error
Mean: 111.8 +/- 1.5 nm
Mode: 83.4 +/- 0.7 nm
SD: 48.1 +/- 3.5 nm
D10: 70.3 +/- 1.9 nm
D50: 99.4 +/- 1.4 nm
D90: 167.5 +/- 3.7 nm
Concentration (Upgrade): 3.08e+10 +/- 8.98e+08 particles/ml
45.9 +/- 1.5 particles/frame
88.5 +/- 6.3 centres/frame
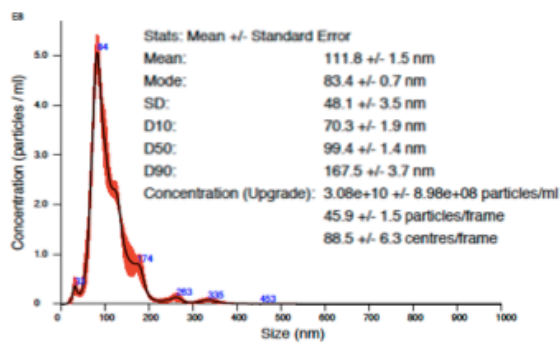
Averaged FTLA Concentration / Size for Experiment:
8-26-19 DiFi Optiprep Coffey Top Load Fractions 1-6 2019-08-26 -
17-34-11
Error bars indicate + / -1 standard error of the mean

Stats: Mean +/- Standard Error
Mean: 118.1 +/- 2.0 nm
Mode: 85.9 +/- 0.7 nm
SD: 49.2 +/- 3.5 nm
D10: 77.1 +/- 0.3 nm
D50: 104.5 +/- 1.8 nm
D90: 174.0 +/- 6.3 nm
Concentration (Upgrade): 3.06e+10 +/- 1.57e+09 particles/ml
46.5 +/- 2.2 particles/frame
51.7 +/- 1.5 centres/frame
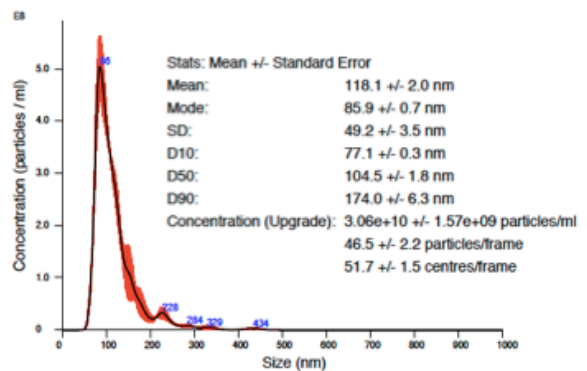
Averaged FTLA Concentration / Size for Experiment:
9-17-19 DiFi Optiprep 7-12 2019-09-17 20-37-51
Error bars indicate + / -1 standard error of the mean

Figure 9. Comparison of Exosome DNA Across Isolation Methods.

(**A**) Exosomes were isolated by three distinct methods as previously described. DNA was obtained from the indicated particle numbers, either with or without Dnase pre-treatment. The presence of nuclease resistant DNA is seen in all fractions tested. Size Exclusion exosomes demonstrate the least sensitivity to nuclease treatment, indicating the purity of the exosome fractions. Statistical significance was evaluated by multiple comparison t-test or two-way ANOVA. ********, $P<0.0001$; ******, $P<0.01$; *****, $P<0.05$; ns, not significant. (**B**) Exosome DNA from each method with and without Dnase pre-treatment was analyzed by capillary electrophoresis (BioAnalyzer). Genomic DNA isolated from matched cell sources were used as positive controls. (**C**) Visual representation of the data shown in B. Black arrow indicates the largest fragment size DNA, seen in cell samples; blue arrow indicates high molecular weight (>2Kb) fragments; red arrow indicates 150bp nucleosome band. (**D**) Representative NTA Nanosight plots for each isolation method, including ultracentrifugation, size exclusion, optiPrep fractions 1-6, and optiPrep fractions 7-12. Plots indicate consistent size distribution and provide particle concentrations for each sample. Samples were diluted 1:100 in sterile water for measurement.

Copy Number Analysis of Exosome DNA Across Different Isolation Methods

Whole genome sequencing was performed on the DNA isolated from exosomes from each method, with or without Dnase treatment. Prior to Dnase treatment, exosomal DNA fragments collected from each method, when pooled, cover the entire genome with copy number variations (CNVs) closely matching those found in matching cellular genomic DNA (**Figure 10 A,B,C**). However, after Dnase treatment, only the size exclusion-generated exosomal DNA produced CNVs that still matched the cellular DNA very closely. Yet, each sample still spanned the entire genome and generally reflected the CNV patterns of the cells of origin (**Figure 10 A,B,C**). This data further suggests that size exclusion is the optimal exosome isolation method for exosomal DNA applications.

Exosomal DNA is Degraded After Membrane Lysis

To ensure that the Dnase-resistant DNA was indeed able to be degraded after losing the protection of the exosome membrane, we treated isolated DNA with Dnase post-lysis. DNA was used from each exosome isolation method, as well as cellular genomic DNA, and all were analyzed by capillary electrophoresis (BioAnalyzer). As expected, the DNA was degraded when treated with Dnase post-lysis, but not pre-lysis of the exosome membrane (**Figure 11**). This supports the notion that the DNA is intraluminal and is protected from degradation by being encapsulated within the exosomal membrane, instead of having degradation prevented by some other mechanism.

10 A

MDA-MB 231

Cell gDNA

Ultracentrifugation — -DNAse / +DNAse

EV DNA

qEV chormatography — -DNAse / +DNAse

Iodixanol gradient — -DNAse / +DNAse

Log2 ratio

Chromosome

10B

SW 620

Cell gDNA

Ultracentrifugation
-DNase
+DNase

EV DNA

Size exclusion
-DNase
+DNase

Iodixanol gradient
-DNase
+DNase

Log2 ratio

10C DiFi

Cell gDNA

Ultracentrifugation · -DNase · +DNase

EV DNA

Size exclusion · -DNase · +DNase

Iodixanol gradient · -DNase · +DNase

Log2 ratio

Figure 10. Exosomal DNA fragments Cumulatively Cover the Whole Genome.

(**A,B,C**) Total exosomal DNA and Dnase-protected exosomal DNA were isolated from three different cell lines using the three methods described previously. DNA from each condition was subjected to whole genome sequencing and analyzed for copy number variations in comparison with genomic DNA isolated from the cells of origin. Exosome DNA isolated by size exclusion provides the best representation of copy number patterns compared with genomic DNA, regardless of DNase pre-treatment. The copy number patterns are shown for MDA-MB 231 (**A**), SW620 (**B**), and DiFi (**C**). Copy number gains are illustrated in red, losses in green, and neutral sites are blue.

Figure 11. Exosomal DNA is degraded after membrane lysis.

DNA was isolated from SW620 cells and exosomes. Exosomes were isolated by ultracentrifugation (UC), Size Exclusion (SE), and OptiPrep density gradient fractionation. For each method, samples were either untreated, treated with Dnase pre-lysis, or treated with Dnase post-lysis. In each case, treatment with Dnase post-lysis degraded the large fragments of DNA while those fragments were preserved with treatment pre-lysis.

11

DNase	Cell		UC			SE			Optiprep		
Pre-lysis	-	-	-	+	-	-	+	-	-	+	-
Post-lysis	-	+	-	-	+	-	-	+	-	-	+
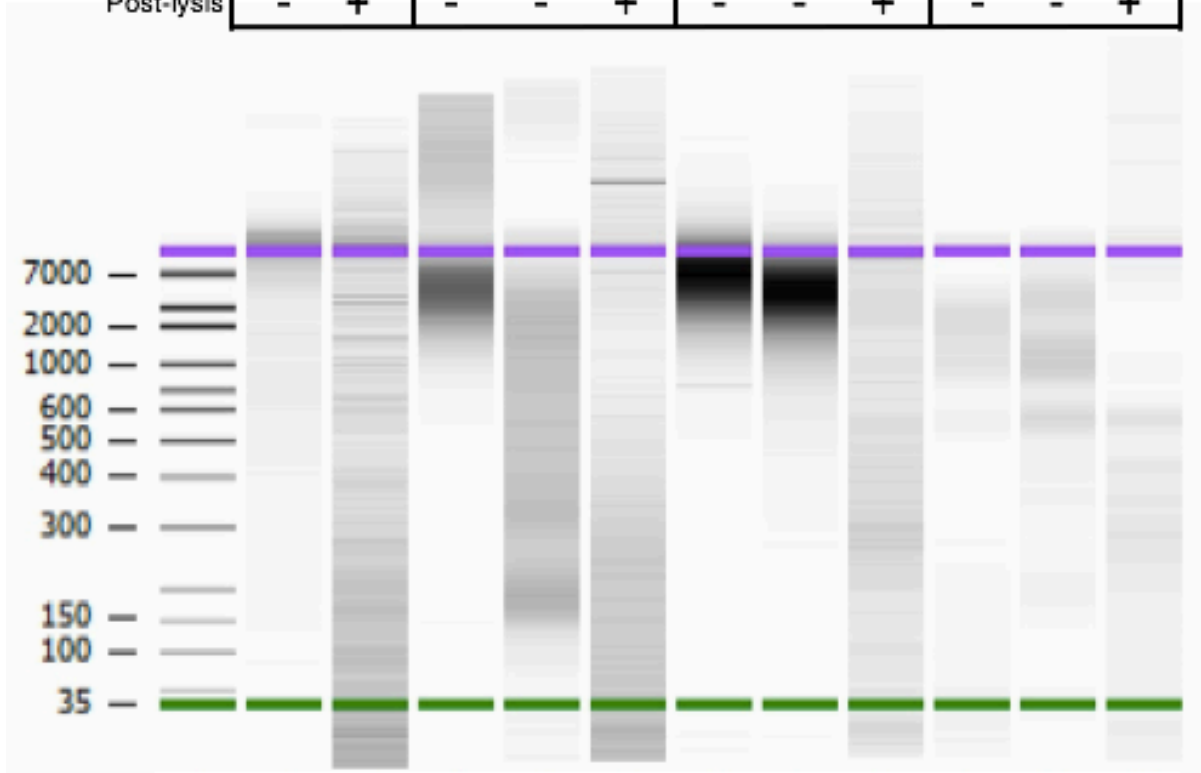
Western Blot Validation of Exosome Markers

To demonstrate the presence of exosomes in the appropriate fractions for size exclusion and the optiPrep density gradients, western blots for canonical exosome markers were run for the samples from each cell line. As observed previously for the density gradients[218], exosome markers CD63, CD81, and CD9 were predominantly seen in fractions 2-6 (**Figure 12 B,D,F**) with flotation density between 0.4-1.18. For size exclusion, the same markers co-localized as expected in the exosome-rich fractions 7-10, while soluble proteins eluted in the non-overlapping distal fractions

(**Figure 12 C,E,G**). Additionally, it is thought that extranuclear genomic DNA remains associated with histones and, at least in part, retains nucleosome organization[282]. Indeed, evidence of a band pattern consistent with approximately 150-bp nucleosome structures is clearly visible in **Figure 9C**. In agreement with this, optiPrep density gradient fractions showed an enrichment of histone 4, especially in the exosomes released by MDA-MB-231 and DiFi cells (**Fig 12 B,D,F**). All size exclusion fractions positive for CD63, CD81, and CD9 were also strongly positive for histone H4 (**Figure 12 C,E,G**). Finally, to ensure that the optiPrep fractions had in fact separated into layers of increasing density from 1-12, absorbance was measured at 340nm wavelength with a plate reader for every gradient, as described elsewhere[278]. A representative plot of flotation density from one gradient is shown in **Figure 12A**.

Flow Cytometry Validation of Exosome Markers

As a secondary method of exosome validation, we took exosomes from each isolation method, with and without DNase treatment, and assessed the samples for the presence of the canonical exosome markers CD9 and CD81 via flow cytometry. Exosomes were bound to aldehyde/sulfate beads (Invitrogen A37304) and incubated with the appropriate primary antibody (**Table 4**). Secondary antibody (Alexa Fluor 488 donkey anti-mouse, Invitrogen A21202) was added and beads were subsequently run on the BD LSR Fortessa flow cytometer for analysis. Exosomes isolated by each method were highly positive for both markers, though the UC isolation samples were not as positive as SEC or density gradient, consistent with the idea that UC is the method with the lowest sample purity (**Figure 13A,B**). Additionally, this data serves to

12A

12B MDA-MB 231
Fraction No

12C MDA-MB 231
Fraction No   Fraction No

12D SW620

12E SW620

12F DiFi

12G DiFi
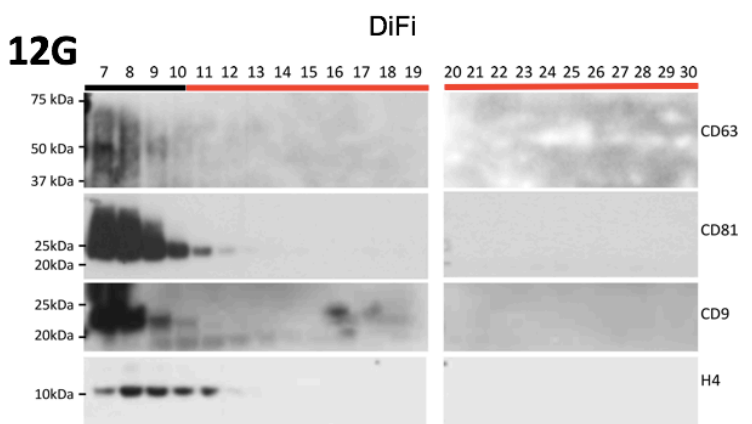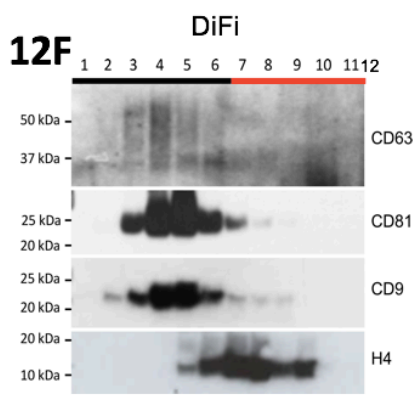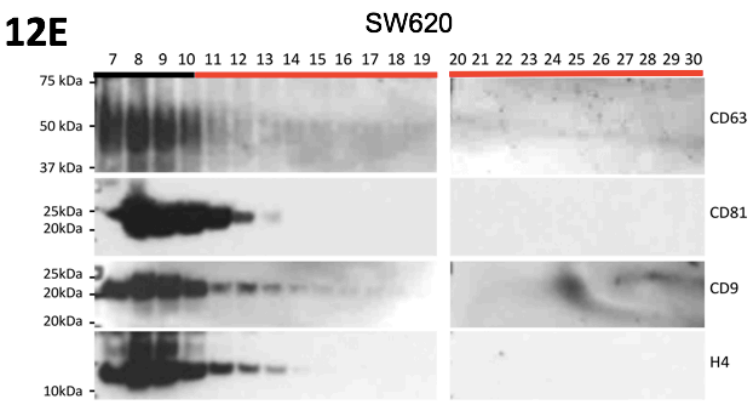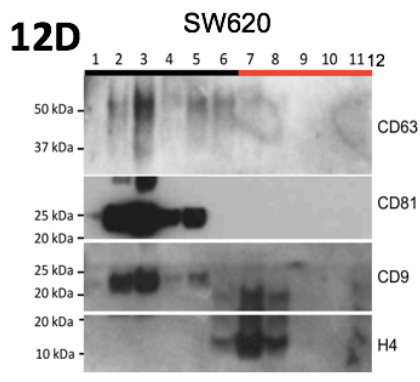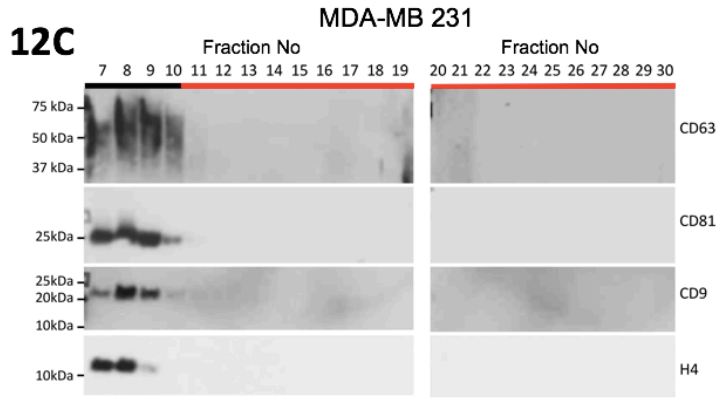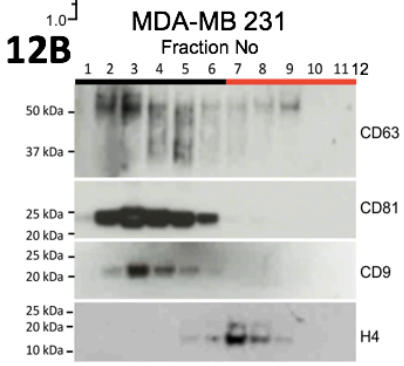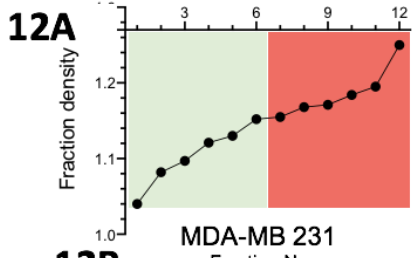
Figure 12. Western Blots for Exosome Markers.

(**A**) Flotation density plot for each optiPrep fraction as measured with a plate reader at 340nm wavelength. The "light" or less dense fractions are indicated by the pale background, while the heavy fractions have a red background. (**B-G**) Exosomes were isolated by ultracentrifugation from three cell lines, MDA-MB 231, SW 620 and DiFi and further purified by Iodixanol gradient ultracentrifugation (OptiPrep) and by size exclusion chromatography (Sephadex columns, qEV, Izon). Fractions were run on polyacrylamide gels and analyzed by western blots probed with antibodies for typical exosomal markers, CD9, CD63 and CD81. To assess the potential distribution of DNA among fractions, the membranes were also probed with antibodies for histone H4. Note the overlap of histone H4 with tetraspanin-positive fractions.

Figure 13. Flow Cytometry Validation of Exosome Markers.

(**A**) Assessment of CD9 tetraspanin expression by flow cytometry. Exosomes from each of the three isolation methods (UC, SE, Density Gradient) with or without DNase treatment prior to lysis were bound to beads and stained for CD9. Unstained cells and isotype controls were used for gating. The blue peak indicates CD9 expression in comparison to the red isotype negative control. All samples tested were positive for CD9, though the UC samples were the least positive (peak shifted left). (**B**) Assessment of CD81 tetraspanin expression by flow cytometry. Results same as for A, just with the CD81 marker, showing consistency in exosome marker expression across all samples.

13A

Staining: CD9   Ultracentrifugation
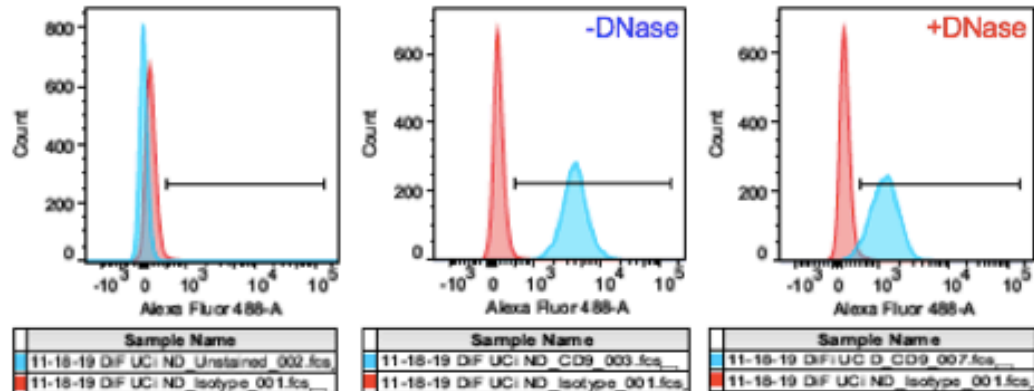
Size Exclusion Chromatography
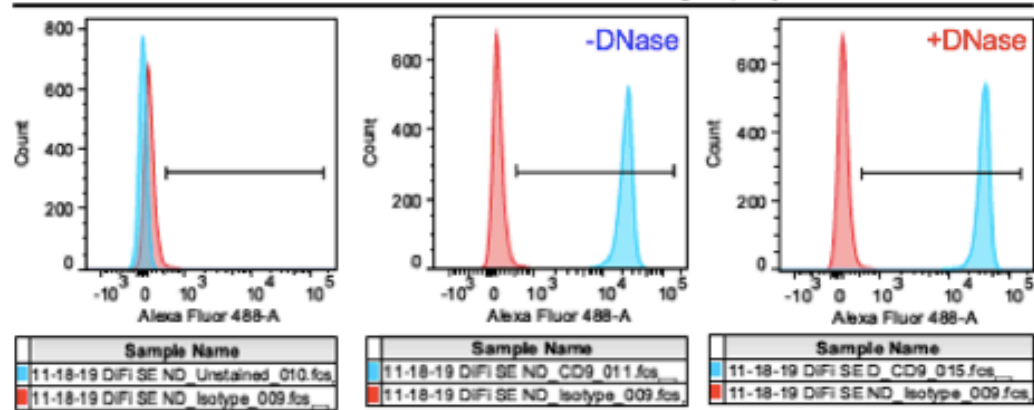
Iodixanol gradient
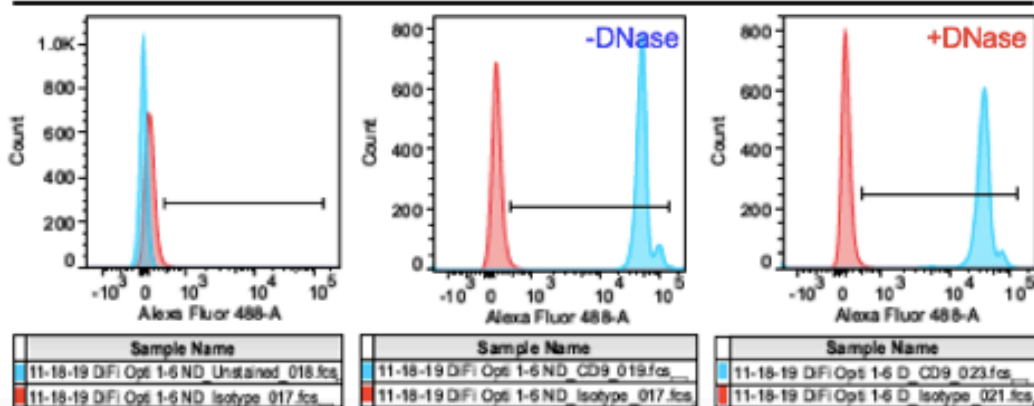
13B Staining: CD81   Ultracentrifugation
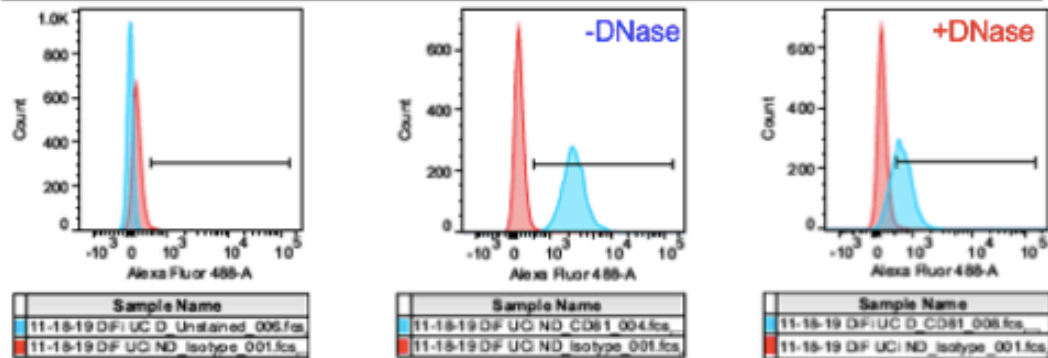
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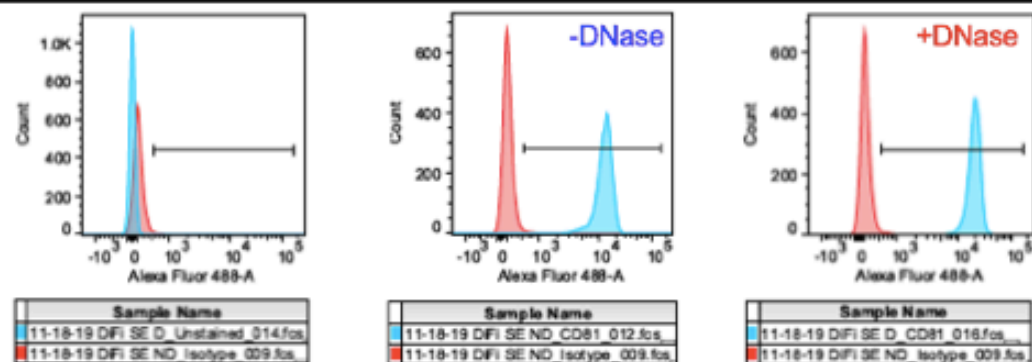
Iodixanol Gradient
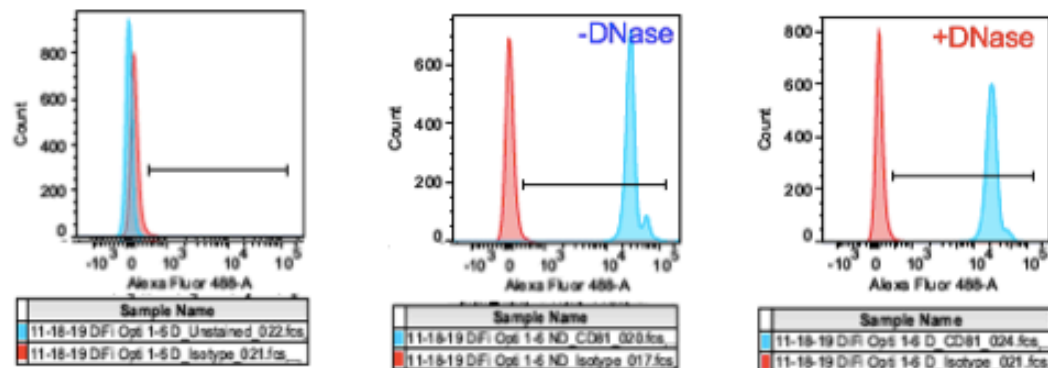
confirm that even after DNase treatment, exosome surface markers remain intact, indicating that they retain membrane integrity.

Argonaute-2 is also associated with exosomes across different isolation methods

Previous studies in our lab and others showed cell-autonomous miRNA-processing capacity of exosomes, due to the presence of intraluminal Argonaute-2 (Ago-2) and other components of RISC complexes, such as Dicer[30,283]. These findings were also questioned in the recent Jeppesen study, which identified Ago-2 in the non-EV fractions of isopycnic Iodixanol gradients[218]. Rigorous analysis of the exosomes released by the same cell lines used in the paper showed, that, like DNA uptake, the association of Ago-2 with the exosomes appeared to be at least partially cell-type dependent. After crude exosomes isolated from DiFi cells were separated by Iodixanol gradient, the exosomes were found in fractions 3-6, as was evidenced by WES of fraction aliquots for the exosome marker Flotillin-1 (**Figure 14A**). Ago-2 was clearly present in fractions 3-5 and formed a distinct secondary peak in fractions 7,8 (non-exosome-containing fractions) (**Figure 14A**). In the case of MDA-MB 231, the exosomes were predominant in fractions 4-8. Ago-2 was significantly more abundant than in DiFi exosomes and segregated in fractions 6-8 and 9-10 (secondary peak, **Figure 14B)**. Size exclusion eluates (fractions 7-10 and every 5th fraction thereafter) presented with Ago-2 in all exosome-containing fractions (**Figure 14C, D**), but only in the case of the DiFi cells were Ago-2 levels sufficiently high to detect a secondary peak in a soluble fraction (fraction 20, **Figure 14C**).

To specifically determine the localization of Ago-2 relative to the exosome membrane, we performed limited proteinase K digestion of crude exosome

preparations as well as exosomes purified by size exclusion chromatography. As a positive control, we used antibodies raised against the large extracellular loop of CD81 (aa 90-200), and antibodies against intraluminal protein Flotillin-1 served as a negative control. The conditions were adjusted so that CD81 was completely digested, while Flotillin remained intact (**Figure 14E**). Using the WES microcapillary immunoassay, we showed that after proteinase K digestion, Flotillin-1 and a significant portion of Ago-2 remained intact, while the extramembrane portion of CD81 was no longer detectable (**Figure 14F**). Interestingly, another protein thought to be associated with exosomes, an autophagy marker LC-3B, was clearly present in crude exosome preparations (**Figure 14G**). However, it was readily removed by proteinase K digestion and not detectable in the size exclusion-purified exosomes, suggesting that at least in this biological context, exosome formation was independent of autophagy.

Conclusion

Here, we have definitively demonstrated the presence of DNA in exosomes using multiple isolation methods, both with and without Dnase treatment. The amount of DNA packaged within exosomes is highly variable depending on the cell line of origin, but cumulatively recapitulates the entire genome. Additionally, exosomes maintain surface marker integrity regardless of Dnase treatment, indicating their membranes are intact. Exosomes also contain proteins such as tetraspanins CD63, CD9, and CD81, as well as Histone H4 and Ago-2.

14A DiFi

Iodixanol gradient fractions

Ab: Flotilin-1 | Ab: Ago-2

L 1 2 3 4 5 6 7 8 9 10 11 12 | L 1 2 3 4 5 6 7 8 9 10 11 12

230, 180, 116, 66, 40, 12 kDa

◄ Ago-2
◄ Flot-1

14B MDA-MB 231

Iodixanol gradient fractions

Ab: Flotilin-1 | Ab: Ago-2

L 1 2 3 4 5 6 7 8 9 10 11 12 | 1 2 3 4 5 6 7 8 9 10 11 12

230, 180, 116, 66, 40, 12 kDa

◄ Ago-2
◄ Flot-1

14C Ab: Flotilin-1

qEV gradient, fractions

Ladder | DiFi | MDA MB 231 | SW230

7 8 9 10 15 20 25 30 | 7 8 9 10 15 20 25 30 | 7 8 9 10 15 20 25 30

kDa 230, 180, 116, 66, 40, 12 kDa

◄ Flot-1

14D Ab: Ago-2

qEV gradient, fractions

Ladder | DiFi | MDA MB 231 | SW230

7 8 9 10 15 20 25 30 | 7 8 9 10 15 20 25 30 | 7 8 9 10 15 20 25 30

kDa 230, 180, 116, 66, 40, 12 kDa

◄ Ago-2

14E

Ab: | CD81 | Flot-1
PrtK | + - | + -

kDa 230, 180, 116, 66, 40, 12 kDa

◄ Ago-2
◄ Flot-1
◄ CD81

14F

Ab: | Flot-1 | CD81 | Ago-2
PrtK | + - + - + - | + - + - + - | + - + - + -

230, 180, 116, 66, 40, 12 kDa

◄ Ago-2
◄ Flot-1
◄ CD81

Method: UC SEC UC | UC SEC UC | UC SEC UC
Cell line: DiFi SW630 | DiFi SW630 | DiFi SW630

14G

Method | UC | SEC
PrtK | + - | + -

230, 180, 116, 66, 40, 12 kDa

] LC-3B

96

Figure 14. Argonaute-2 is present in size exclusion and density gradient isolated exosomes.

(**A,B**) Exosome fractions were isolated by optiPrep density gradient from the cell lines DiFi (A) and MDA-MB 231 (B). Protein extracts were prepared and assessed for Ago-2 content by WES microcapillary immunoassay. Similarly loaded extracts were probed with antibodies for Flotillin-1 as a control to assess protein loading. Differences are observed in Ago-2 incorporation between individual cell lines. (**C, D**) Crude Exosome isolates were fractionated by size exclusion chromatography for the indicated cell lines. Equal volume from each fraction was loaded onto a WES cassette and probed with antibodies against Flotillin-1 (C), or Ago-2 (D) to assess exosome protein content. Ago-2 was present in flotillin-positive fractions containing exosomes. (**E**) Proteinase k treatment was optimized to completely degrade exosome membrane marker CD81, representative of external exosome-associated proteins, but not flotillin-1, representative of intraluminal exosome proteins. (**F**) Proteinase k treated exosomes from the indicated cell lines and isolation methods demonstrated a decrease in Ago-2 but still clearly showed some remaining, indicating that at least a portion of the Ago-2 associated with exosomes is inside the intraluminal space. (**G**) The autophagy marker LC-3B is present in ultracentrifugation but not size exclusion-isolated exosomes, and is degraded by proteinase k treatment. This suggests that LC-3B is not contained within these exosomes, indicating they were generated in a process independent of autophagy. These experiments were performed in collaboration with Laura M. Snowden and Olga Volpert.

CHAPTER 4

EXOSOME DNA METHYLATION

Introduction

Despite the relative paucity of characterization of exosomal DNA compared to other exosome cargo, several studies point to the great potential of exosomal DNA as a biomarker for diagnosis and monitoring of cancer and other diseases. Indeed, multiple groups have been able to isolate exosomal DNA from patient samples and identify specific polymorphisms and pathogenic mutations in those specimens [105–107,162]. There has been very little investigation, however, into determining whether or not epigenetic modifications can be detected on exosomal DNA and if so, whether they are representative of the state of the donor epigenome. Studies over the past several decades have made it increasingly obvious that mutations alone do not comprehensively define many complex disease states, such as cancer[284]. Epigenetic profiles, especially methylation patterns, have also been demonstrated to provide valuable clues for the diagnosis and treatment of disease, as methylation changes can be as effective as mutations in disease pathogenesis[285,286]. It has now been well established that differential methylation can produce clinically relevant changes in gene expression, with major roles in the development and outcome of several diseases[287]. Thus, developing new methods for the detection of aberrant methylation profiles or patterns, especially those linked to disease progression, would be invaluable.

Perhaps because the field of exosomal DNA research is relatively nascent, there is a distinct lack of information with regards to exosomal DNA methylation. The existing epigenetics-related exosome studies have instead focused on the ability of exosomes to perpetrate epigenetic changes in recipient cells by delivering protein

cargo capable of enhancing methylation or demethylation [288]. To date, the number of published studies analyzing the potential of exosomal DNA for methylation detection remains minimal. In the first study, done by the Lyden group, a DNA dot blot probed for anti 5'-methyl cytosine and demonstrated comparable levels of methylation on cytosines in exosomal DNA and in cellular genomic DNA[47]. Another group interested in gastric cancer demonstrated similar methylation profiles of the BARHL2 locus, LINE1, and SOX17 genes in the exosomal DNA from the gastric juice and gastric cancer cells of patients by site-specific bisulfite PCR and bisulfite pyrosequencing[289]. Beyond these studies, no comprehensive analysis have been performed investigating the extent, patterning or potential function of exosomal DNA methylation. We hypothesized that exosomal DNA is methylated, and that the methylation patterns on exosomal DNA recapitulate those of the cell of origin. The aim of this section is to systematically address the correlation between methylation patterns in the exosomal DNA and cells of origin. These findings will provide solid foundation for potential use of exosomal DNA methylation analysis as a diagnostic strategy. This is especially relevant for cancers notorious for late diagnosis, such as pancreatic ductal adenocarcinoma (PDAC).

Characterization of PDAC Cell Line Exosome DNA Methylation

Exosomal DNA is Methylated

Initial experiments aimed to determine whether exosomal DNA is methylated (**Figure 15**). For that purpose, genomic DNA was isolated from cells and matched exosomal DNA were collected by ultracentrifugation. Six tumorigenic PDAC lines

(Panc1, BXPC3, Capan-1, T3M4, MiaPaCa2, and PSN1) and two non-tumorigenic

lines (HPNE and HPDE) were used. The DNA was then purified using the method

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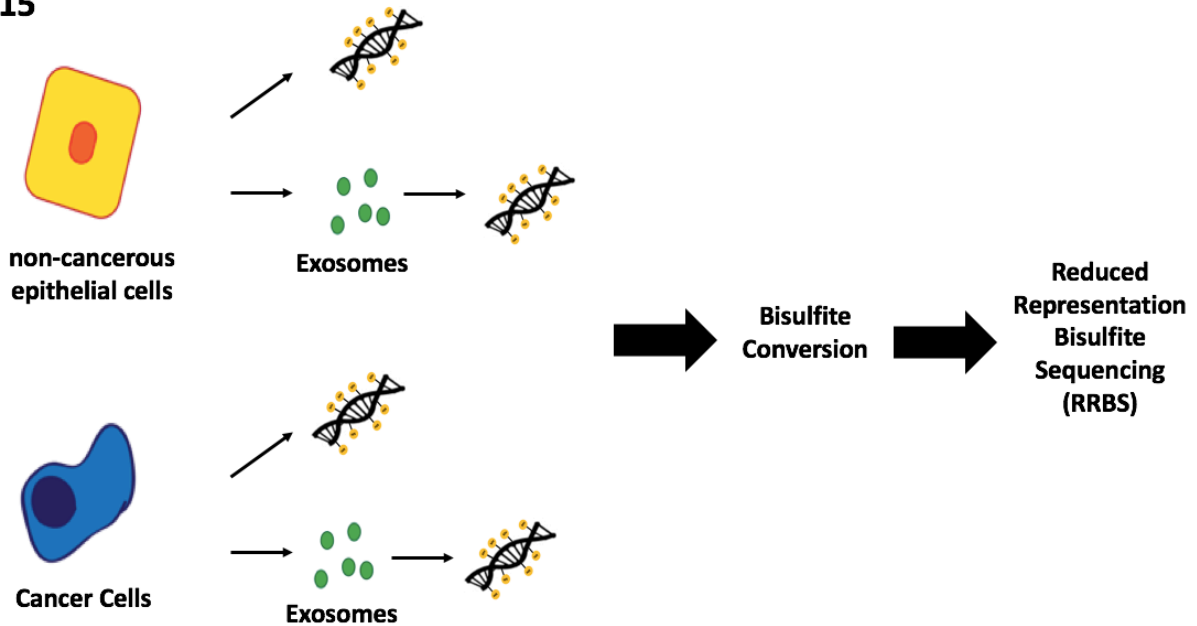
Figure 15. Schematic of methylation detection experiment design

Nuclear genomic and exosomal DNA were isolated from multiple cell lines, both

tumorigenic and non-tumorigenic, and subsequently underwent bisulfite conversion,

library formation, and reduced representation bisulfite sequencing (RRBS).

described previously (DNeasy Mini Kit, Qiagen with minor modifications) and

subjected to bisulfite conversion, a process that changes unmethylated cytosines into

uracils, which are subsequently converted to thymidines during PCR amplification.

This method allows for the identification of methylated and unmethylated cytosines

when sequenced. Samples then underwent reduced representation bisulfite

sequencing (RRBS). While genome-wide cytosine methylation analysis is possible,

methylated cytosines tend to cluster in CpG dinucleotide-rich areas called CpG islands, often in the promoter regions of genes, that collectively span a very small portion of the genome. RRBS targets CpG rich regions using a primer library, which then generates site-specific fragments. This method also only requires small amounts of DNA, which is useful for exosomal DNA applications[290,291]. During analysis, the amplified fragments, or reads, are mapped to specific regions of DNA using reference sequences. The number of C-to-T conversions is then quantified for all of the mapped reads, generating a beta value for methylation at each site. Samples can then be compared in a site-specific manner between conditions, in this case between exosomal and matched cellular DNA.

A custom bioinformatics analysis pipeline was generated for this application through a collaboration with Dr. Kunal Rai's laboratory at MD Anderson Cancer Center. Analysis of the first cell line tested, Panc1, demonstrated site-specific methylation patterns for both the exosome and cellular nuclear DNA. A Pearson correlation coefficient was calculated and exosome DNA methylation was observed to be highly concordant with the nuclear DNA methylation (**Figure 16**). Interestingly, when differences were observed, exosomal DNA was more often hypermethylated (n=3510 genes) than hypomethylated (n=27 genes) compared to the nuclear DNA, suggesting that perhaps exosomal DNA is protected from demethylating enzymes when it is packaged inside exosomes. Subsequently, the analysis was expanded to include multiple cell lines, and the quality control of sequencing data showed sufficient quality, read depth, and alignment to produce reliable results (**Figure 17 A-C**). Therefore, we for the first time provide conclusive reliable and reproducible evidence

16
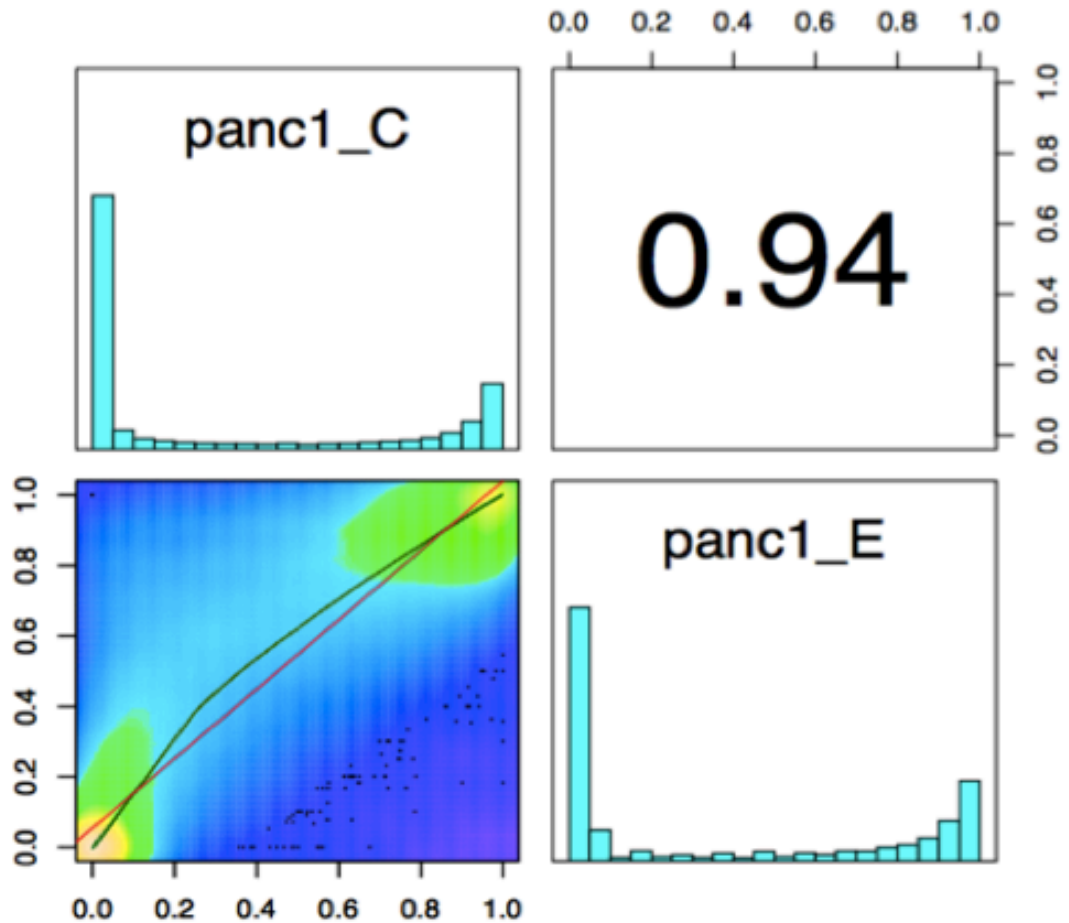
Figure 16. High correlation between cell and exosome methylation patterns.

Correlation of methylation patterns between cell gDNA end exosome DNA isolates was very high, with a Pearson Correlation coefficient of 0.94. These samples are representative of what was observed across multiple cell lines. Exosome samples were treated with Dnase prior to lysis. This analysis was performed by Dr. Tommy Tang in Dr. Kunal Rai's laboratory.

17 A

17 B
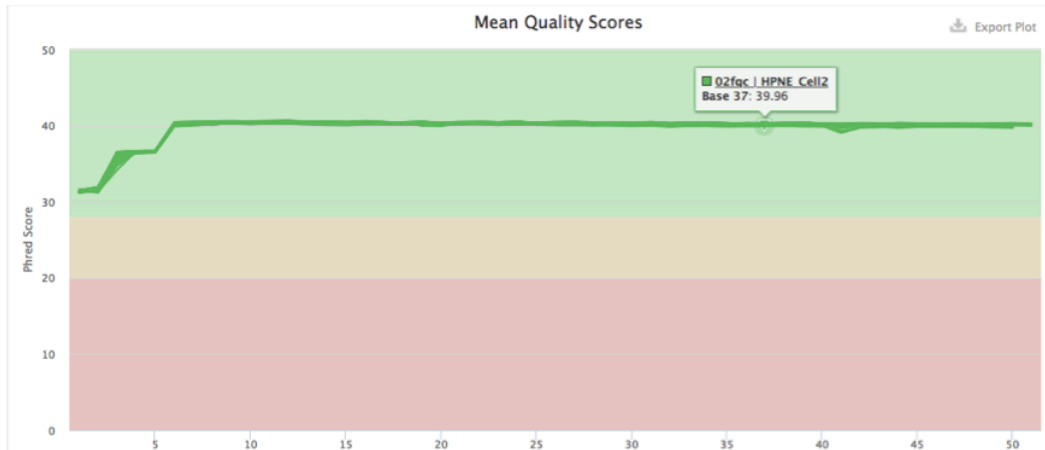
17 C
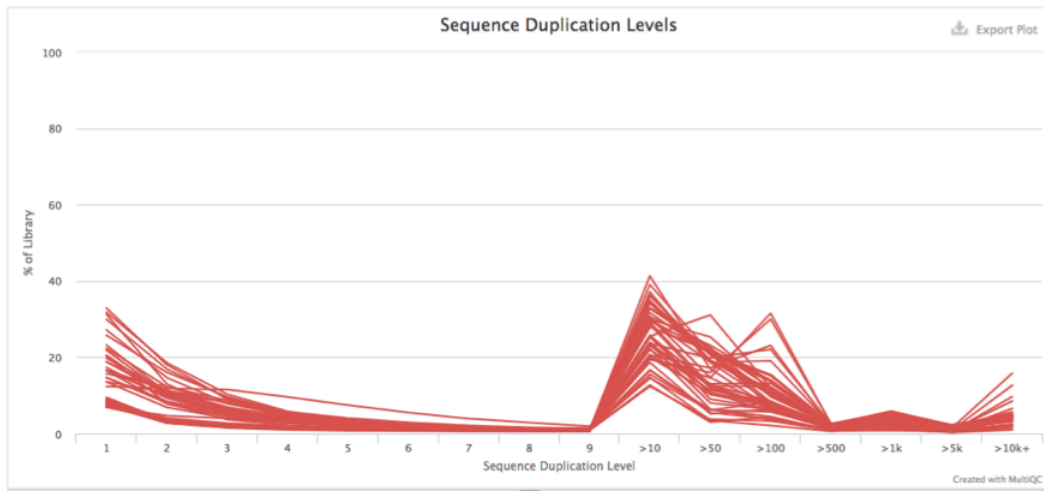
Figure 17. Quality control of sequencing data

(**A**) Alignment rates were high among all samples. (**B**) base call quality is high, indicating there are few sequencing errors and the reads can be accurately analyzed. (**C**) There is a high number of duplicates, which is expected as a result of the primer library used in RRBS to amplify specific sites of the genome.

that exosomal DNA is methylated and harbors highly conserved methylation patterns from the cell of origin.

Exosome DNA Methylation Patterns Reflect Methylation of the Cell of Origin

We then aimed to determine whether the methylation patterns seen in the pooled exosome samples were sufficiently unique to provide a cell line-specific signature. To achieve this, broad Pearson Correlation analyses were performed to compare each cell and exosome sample from a given cell line to those from others (all done in biological triplicates, 6 samples total per line, 3 cell and 3 exosome). As illustrated in **Figure 18**, methylation patterns identified on cell gDNA and exosomal DNA are closely matched when compared within a single cell type. However, if samples are compared between cell lines, the correlation significantly decreases. These data suggest that exosomal DNA not only recapitulates the methylation patterns of the cell of origin but also carries a cell-specific methylation signature with high fidelity.

Exosome DNA Methylation Patterns Can Specifically Identify the Cell of Origin

Since we discovered that methylation patterns found on exosomal DNA are distinct and correlated extremely well with the methylation patterns observed on the

18
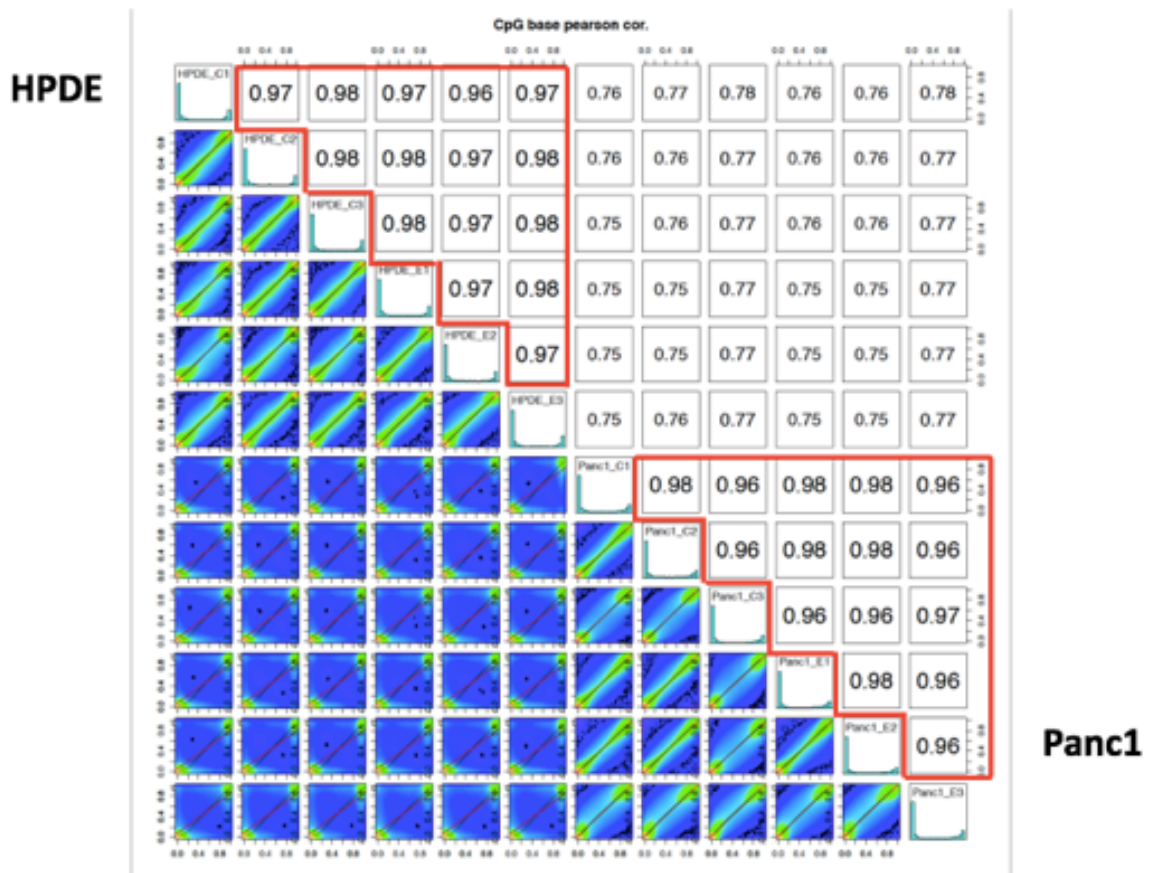
Figure 18. There is high methylation pattern correlation among samples from the same cell line. Example of a comparison between genomic cell and exosome DNA for two lines, Panc1 and HPDE. As outlined in red, samples from the same cell type, regardless of origin, cluster together. Samples from different cell lines have markedly lower correlation. This analysis was performed by Dr. Tommy Tang in Dr. Kunal Rai's laboratory.
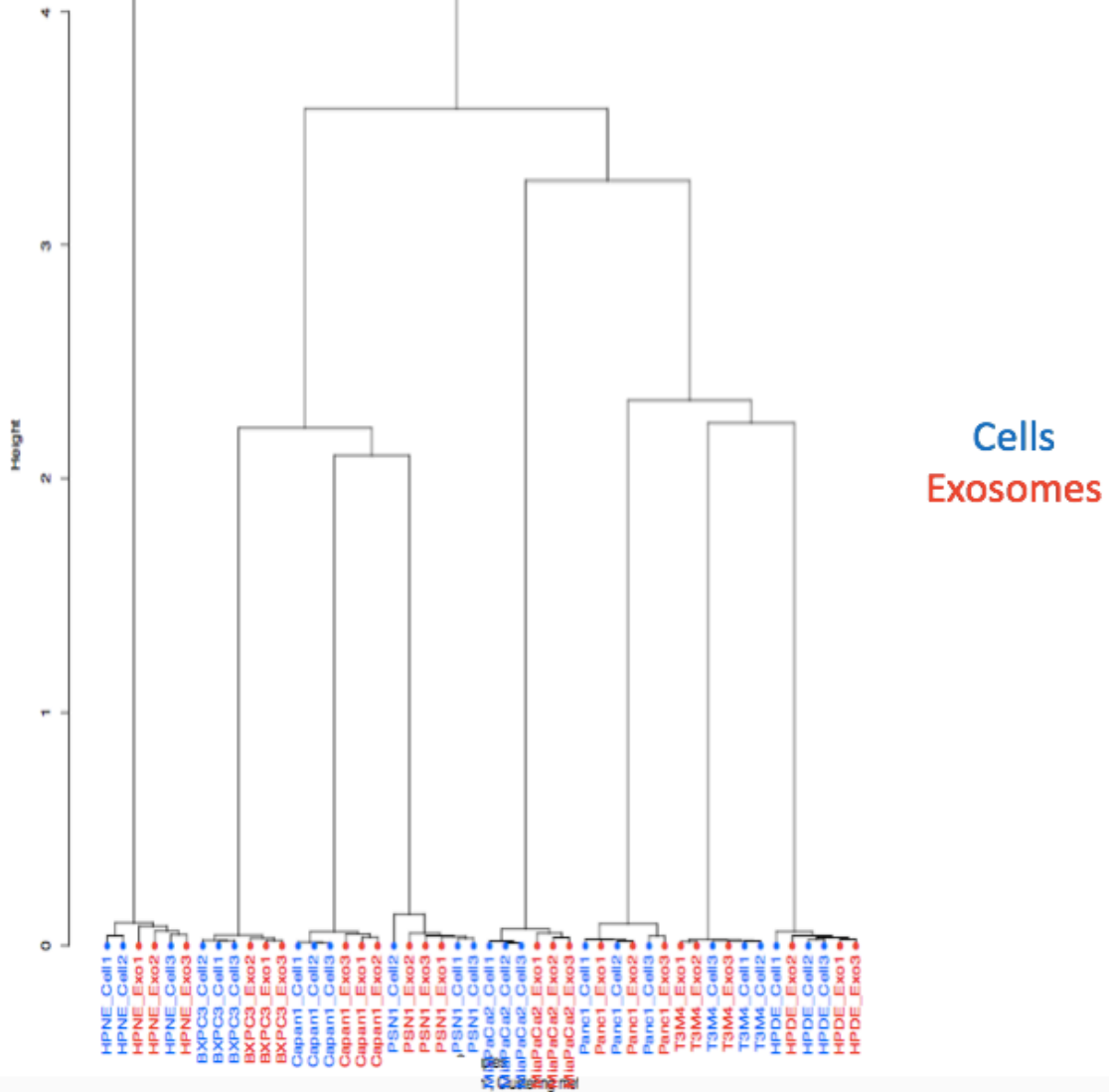
Figure 19. Clustering Analysis for All Eight Cell Lines.

All biological replicates of cellular and exosomal DNA from each individual cell line cluster together, based on methylation pattern comparison. The segregation of exosome and nuclear DNA according to the cell line of origin indicates that exosomal DNA carries a cell line-specific signature. This analysis was performed by Dr. Tommy Tang in Dr. Kunal Rai's laboratory.

genomic DNA of the cell of origin, we then performed a clustering analysis across all cell and exosome samples. (**Figure 19**). This assay demonstrated that both the cellular gDNA and exosomal DNA from each individual cell line cluster together, and are unique and separate from any other line. This implies that exosome-derived methylation signatures could be used for the detection of cancer-specific markers in patients.

Methylation Marks Detected on Exosome DNA Span All Chromosomes

Since RRBS employs sequencing library generation using primers specific to known frequently methylated areas of the genome, it is possible to perform a post-sequencing analysis of genome read coverage. We demonstrate here that exosomal DNA methylation patterns are representative of every chromosome in the genome in every sample. This finding provides conclusive evidence that pooled exosomal DNA preparations can yield genome-wide methylation data, with no overt biases for any specific loci or hotspots (**Figure 20)**.

Exosome DNA Can be Used to Investigate Site-Specific Methylation Changes

We next tested the possibility of probing the exosomal RRBS sequencing datasets for the presence of cancer-specific methylation changes when comparing the PDAC and non-tumorigenic cell lines. An in-depth literature search identified over 75 functionally diverse genes differentially methylated in PDAC (**Table 7**). The top six were chosen for site-specific analysis (**Table 8**). The Broad Institute Integrative Genomics Viewer (IGV) software was used to visualize methylation levels in the promoter regions of the genes of interest (**Figure 21A**). The percentage of methylated
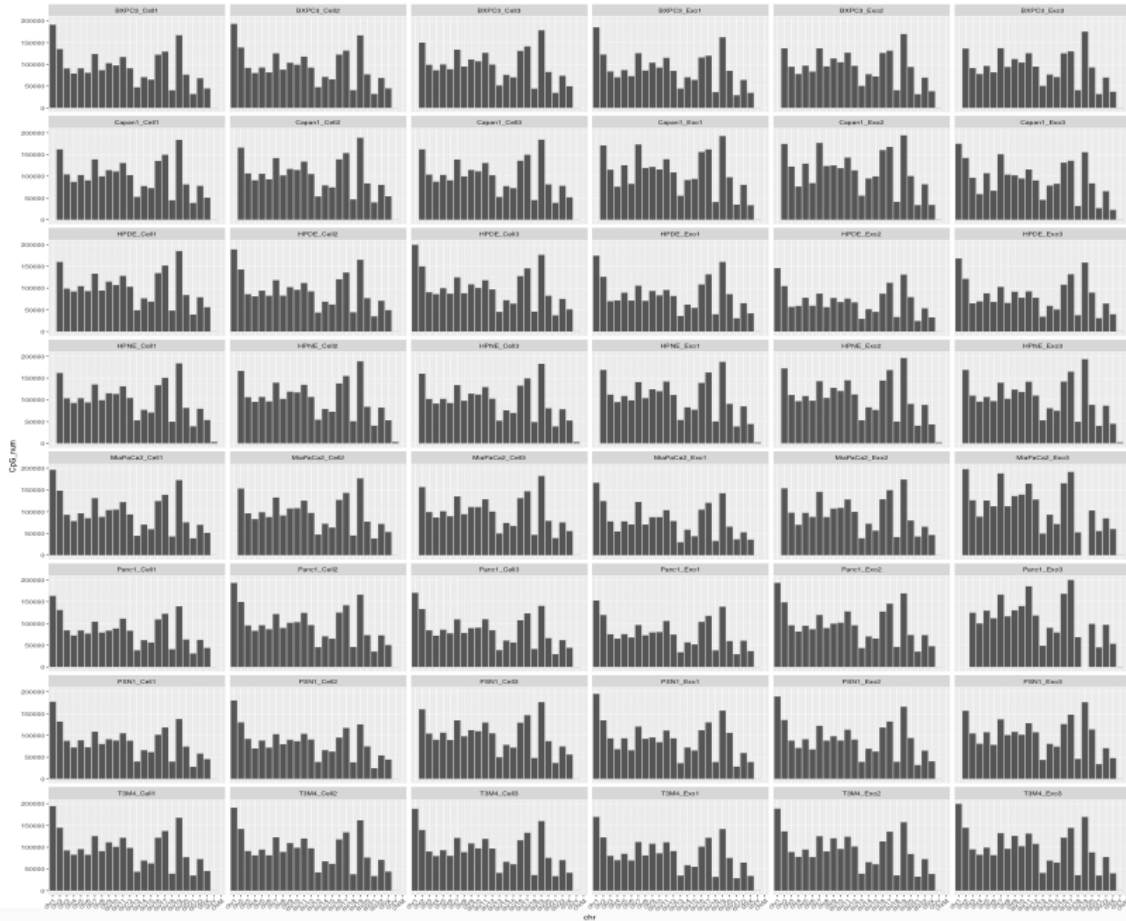
Figure 20. Methylation marks from cells and exosomes span all chromosomes.

Representative analysis of reads per chromosome for each sample (3 cellular genomic and 3 exosome DNA samples for each cell line, 8 cell lines total). Each plot represents a single sample, and each gray bar indicates a single chromosome. Every chromosome is represented in every sample. This analysis was performed by Dr. Tommy Tang in Dr. Kunal Rai's laboratory.

Table 7. Genes Differentially Methylated in PDAC.

Gene	Function	PDAC Alteration
PENK	growth suppressive; neuropeptide precursor	hypermethylation
UCHL1	ubiquitin hydroxylase	hypermethylation
MDF-1	Glycogen Metabolism	hypermethylation
NPTX2	Neuronal Transport, proliferation, invasion	hypermethylation
SPARC/ON	Cell cycle progression, migration, cell matrix interaction	hypermethylation
RPRM (reprimo)	p53 dependent G2 arrest mediator	hypermethylation
BNIP3	hypoxia-induced cell death, chemoresistance	hypermethylation
miR9-1	miRNA translation control	hypermethylation
SERPINB5 (maspin)	Regulation of cell motility & cell death	hypomethylation
CCND2 (cyclin D2)	Cell cycle control	hypermethylation
ZNF415	Transcriptional Regulation	hypermethylation
CLDN4	Cell adhesion/invasion	hypomethylation
SFN (stratifin, 14-3-3 sigma)	p53 induced G2/M cell cycle arrest	hypomethylation
LCN2 (Lipocalin2)	Epithelial differentiation	hypomethylation
TFPI2	Serine protease inhibitor, growth, invasion	hypermethylation
CNTNAP2	Higher Cortical Function	hypermethylation
CDKN1C/p57	Cyclin-dependent kinase inhibitor	hypermethylation
SIP1	Assembly of spliceosomal snRMP	hypermethylation
ELOVL4	Fatty Acid Synthesis	hypermethylation
TFF2 (trefoil factor 2)	Secretory Peptide/epithelial repair	hypomethylation
FOXE1	Thyroid transcription factor	hypermethylation
S100P	Cell cycle progression/differentiation	hypomethylation
RARB	Cell growth control	hypermethylation
S100A4	Motility, Invasion, Tubulin Polymerization	hypomethylation
CDKN2A/p16	Cyclin-dependent kinase inhibitor	hypermethylation
MSLN (mesothelin)	Cell surface antigen/cell adhesion	hypomethylation
SOCS1	JAK/STAT inhibitor	hypomethylation
PSCA (Prostate Stem Cell Antigen)	Cell surface antigen/cell differentiation	hypomethylation
CADM1/TSLC1	Cell-Cell and Cell-Matrix interactions	hypermethylation
MLH1	DNA Repair	hypermethylation
NDRG1	Hormone Responses, Cell Growth, Differentiation	hypermethylation
CDH1	Cell adhesion/invasion	hypermethylation
RASSF1A	tumor suppressor-Ras associated domain	hypermethylation
SOX15	transcriptional Regulation; embryonic development	hypermethylation
KLF10	Transforming growth factor	hypermethylation
HOPX	homeodomain, cardiac growth/development, transformation	hypermethylation
CLDN5	integral membrane protein, tight junction	hypermethylation
SFRP	Wnt signaling modulator	hypermethylation
SARP2 (SFRP2)	apoptosis mediator, wnt/frizzled interference	hypermethylation
LHX1	Transcription factor, differentiation	hypermethylation
WNT7A	Wnt signaling pathway, development	hypermethylation
TJP2	tight junction protein	hypermethylation

110

Table 7 Continued.

Gene	Function	PDAC Alteration
CDH3	cadherin; cell adhesion	hypermethylation
ST14	epithelial membrane activator for protases and growth factors	hypermethylation
miR132	electron transport, STP synthesis	hypermethylation
FOXA 1/2	transcriptional activator, metabolism, pancreatic differentiation	hypermethylation
ARID1B	SWI/SNF remodeling complex protein, cell cycle activation	hypermethylation
Dkk3	embryonic development, wnt signalling tumor suppressor	hypermethylation
Cldn18	claudin; tight junctions, MAPk regulated	hypermethylation
TNFRSF10C	TNF family receptor; p53 regulated DNA damage inducible; protects from TRAIL-induced apoptosis	hypermethylation
RUNX3	Transcription factor, tumor suppressor	hypermethylation
PTCHD2/DISP3	neuronal proliferation, differentiation	hypermethylation
SOX17	embryonic development, cell fate determination, transcriptional regulator	hypermethylation
NXPH1	adhesion between dendrites and axons	hypermethylation
EBF3	regulation of cell cycle arrest and apoptosis	hypermethylation
RELN	cell positioning and neuronal migration in development; metabolism, PI3K-Akt	hypermethylation
VAV1	GEF for Rho family GTPases; T-and B-cell development/activation; transcription & replication	hypomethylation
MUC4	Mucin; integral membrane glycoprotein-metabolism, ErbB2 binding, ECM	hypomethylation
ABCB1/MDR1	resistance; ATP-dependent drug efflux pump	hypomethylation
ABCC1/MRP1	resistance; multispecific organic anion transporter	hypomethylation
ABCG2/BCRP	resistance; xenobiotic transporter	hypomethylation
BRCA1	tumor suppressor, genomic stability, transcription, dsb repair, recombination	hypermethylation
PGR	progesterone receptor	hypermethylation
SYK	proliferation, differentiation, phagocytosis; tumor suppressor	hypermethylation
VHL	ubiquitination and degradation of HIF; tumor suppressor	hypermethylation
DAPK1	death associated protein kinase; mediator of gamma interferon induced cell death	hypermethylation
ESR1 promA	estrogen receptor	hypermethylation
MGMT	methyltransferase; DNA repair	hypermethylation
MUC2	mucin; forms an insoluble mucous barrier	hypermethylation
MYOD1	transciption factor, muscle cell differentiation, cell cycle arrest	hypermethylation
CDKN2B	cdk inhibitor, growth regulator, controls cell cycle G1 progression	hypermethylation
PGK1	angiogenesis	hypermethylation
RB1	Tumor suppressor, cell cycle regulation	hypermethylation
hsa-mir-9-1	Cell differentiation, electron transport, ATP synthesis	hypermethylation
CACNA1G	T-type calcium channel	hypermethylation
TIMP3	MMP inhibitor	hypermethylation
THBS1	Thrombospondin; mediated cell-cell and cell-matrix interactions	hypermethylation
CDH11	cadherin; calcium dependent cell-cell adhesion	hypomethylation
APC	tumor suppressor, wnt signalling antagonist, migration, adhesion, transcription activation, apoptosis	hypermethylation
WWOX	tumor suppressor, apoptosis	hypermethylation
CDH13	cadherin; negative regulator of axon growth, protects vascular endothelial cells from apoptosis	hypermethylation
DUSP6	protein phosphatase, inactivates ERK2	hypermethylation
HHIP	Hedgehog-interacting protein; development, cAMP signalling	hypermethylation

Table References[289,292–298]

Table 8. Genes Selected for Site-Specific Analysis

Gene	Function	PDAC Alteration
ELOVL4	Fatty Acid Synthesis	hypermethylation
BNIP3	hypoxia-induced cell death, chemoresistance	hypermethylation
ZNF415	Transcriptional Regulation	hypermethylation
TFPI2	Serine protease inhibitor, growth, invasion	hypermethylation
RASSF1A	tumor suppressor-Ras associated domain	hypermethylation
CLDN5	integral membrane protein, tight junction	hypermethylation

reads was determined on a single-base pair level, for all CpG dinucleotides within the promoter regions (**Figure 21B**). For the six genes of interest, the average overall promoter methylation was determined for both the genomic and exosomal DNA samples from all eight cell lines used in the study (**Figure 22**). Similar methylation patterns were maintained in exosomal/cellular gDNA in tumorigenic and non-tumorigenic groups, with the non-tumorigenic lines generally demonstrating promoter hypomethylation and the tumorigenic lines presenting with promoter hypermethylation, consistent with published information for each of the gene methylation states (PDAC vs. healthy tissue). There is some variation within the groups on a cell-line specific basis, which is to be expected due to variability due to inherent cell culture heterogeneity. These findings indicate that exosomal DNA can be utilized to identify site-specific methylation changes with good reliability, which could be utilized for discovery or precision medicine.

Exosome DNA Can Detect Decreased Methylation in Cells Treated With 5AZA

Since demethylating agents are being utilized in cancer therapy[299], we chose to investigate whether exosomal DNA is suitable for the detection of large-scale methylation changes resulting from treatment with 5-Azacitidine (5AZA), a chemical

21A
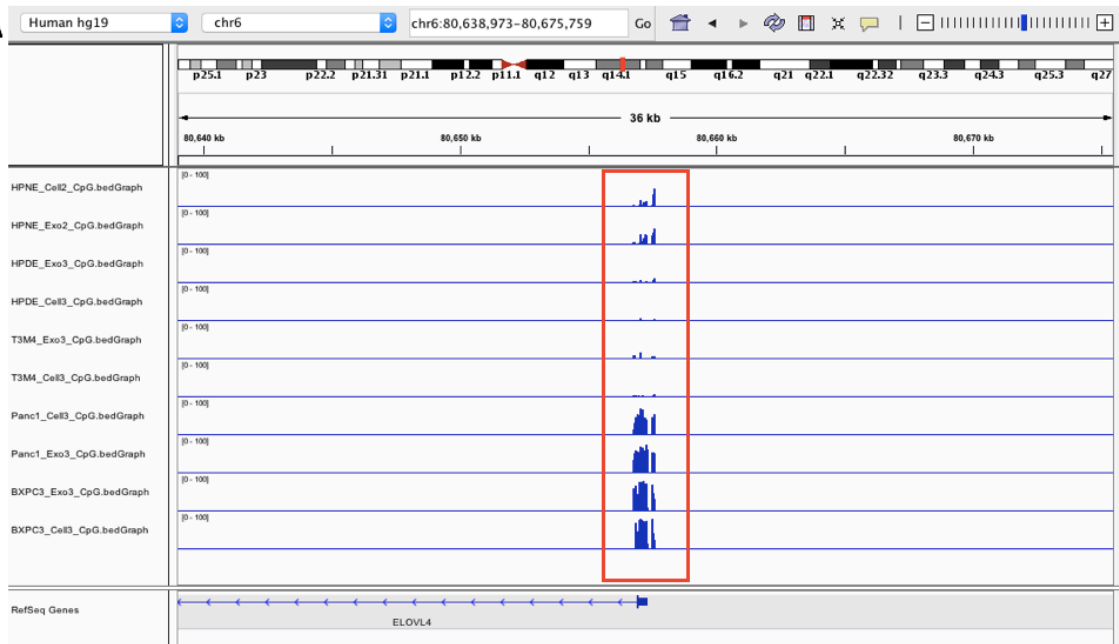
Gene: ELOVL4

21B
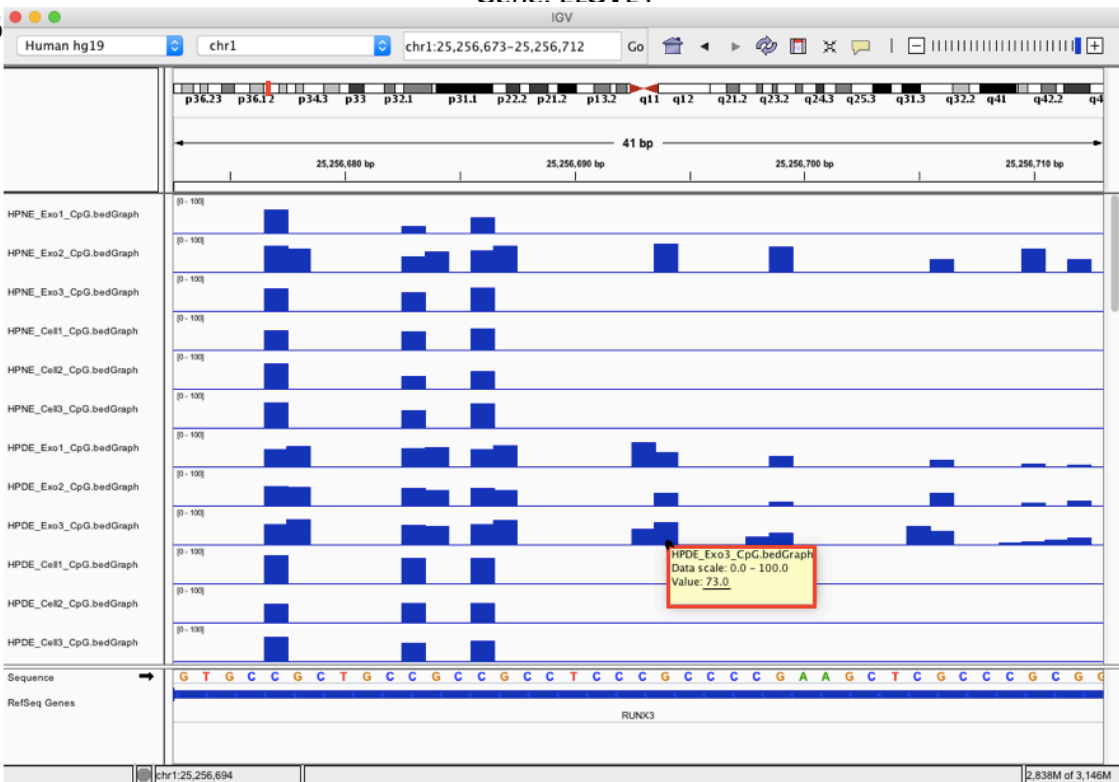
Figure 21. Use of Broad IGV Software to assess site-specific methylation.

(**A**) Visualization of methylation (blue bars) in the promoter region (red box) of the gene ELOVL4 across cellular and exosomal DNA samples. This strategy allows for quantification of methylation in specific regions of genes of interest. (**B**) Methylation rates of individual CpG dinucleotide sites shown as percentage of methylated reads at each site in each sample. Allows for an assessment of the degree of methylation at each site in a given sample.

Figure 22. Average Overall Promoter Methylation in Genes Frequently Hypermethylated in PDAC.

(**A-F**) Six genes established as frequently hypermethylated in pancreatic cancer (ELOVL4 (A), BNIP3 (B), ZNF415 (C), TFPI2 (D), RASSF1A(E) and CLDN5 (F)) were assessed for percentage of promoter methylation levels in both cellular genomic and exosome DNA samples. Non-tumorigenic cell lines (HPNE, HPDE) are shown on the left and tumorigenic ones (Panc1, T3M4, BXPC3, MiaPaCa2, Capan1, PSN1) are on the right in each graph. Generally, the non-tumorigenic samples from both genomic and exosomal DNA exhibited hypomethylation while tumorigenic samples showed hypermethylation, consistent with PDAC methylation changes reported in the literature.

22A Average Promoter Methylation: ELOVL4
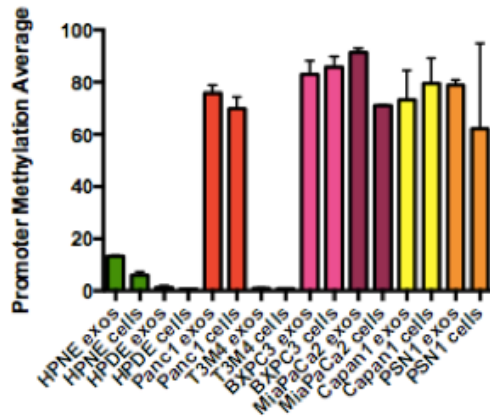
22B Average Promoter Methylation: BNIP3
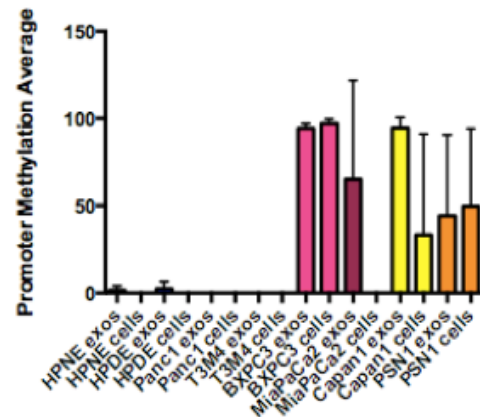
22C Average Promoter Methylation: ZNF415
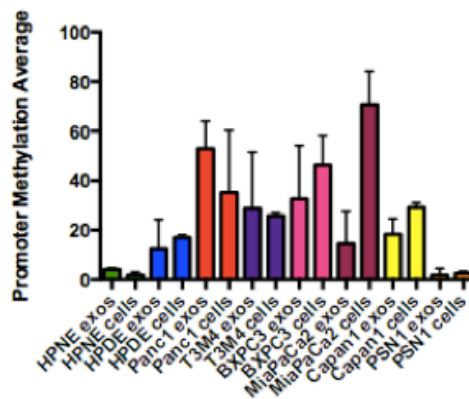
22D Average Promoter Methylation: TFPI2
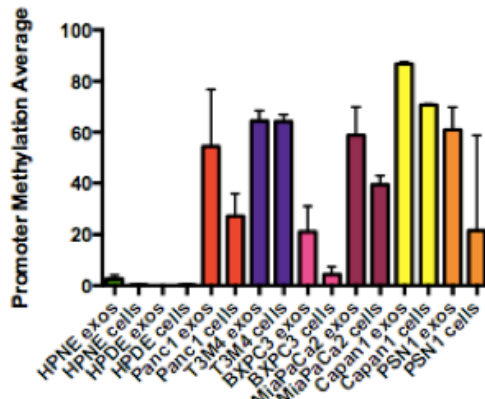
22E Average Promoter Methylation: RASSF1A
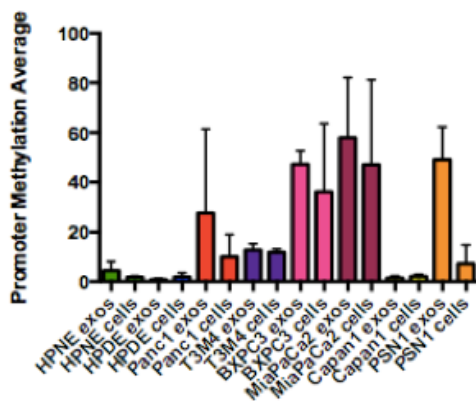
22F Average Promoter Methylation: CLDN5
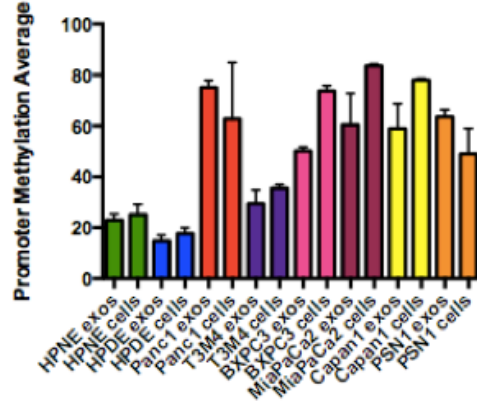
analogue of the nucleoside cytidine that, at low doses, inhibits DNA methyltransferase and causes DNA hypomethylation[300]. Panc1 cells were treated with increasing doses of 5AZA to generate a dose-response curve and determine the optimal non-lethal demethylating dose (**Figure 23A**). We then utilized this dose in a high- sensitivity fluorescence-based assay to detect methylated DNA using a 5-methylcytosine antibody in Panc1 nuclear gDNA and exosome DNA from cells with and without exposure to 5AZA. Preliminary experiments have shown that decreased methylation levels could be detected in exosomes isolated from 5AZA-treated cells (**Figure 23B**). This highlights a potential application for using patient-derived exosomes to monitor the *in vivo* efficacy of demethylating agents.

Figure 23. Exosomes can be used to detect drug-induced methylation changes. (**A**) Dose-response curve to determine the optimal non-toxic dose of 5-AZA in Panc1 cells (red box). (**B**) Methylation levels were assessed in nuclear genomic DNA and exosomal DNA from cells with or without 5AZA treatment using the Abnova Methylated DNA Quantification Kit. Formulas for methylation level calculations are shown. Methylation levels are decreased in both genomic and exosomal DNA samples after 5AZA treatment, indicating that exosomal DNA reflects rapid methylation changes due to drug treatment.

23A

Dose response curve for DNA demethylation (Panc-1 cells)

$$\text{Methylation\%} = \frac{\text{RFU(sample-NC)}/0.41}{\text{RFU(PC-NC)}\times 10} \times 100\%$$

NC=negative control
PC=positive control
0.41=GC content in the human genome
10= Sample/PC DNA ratio.
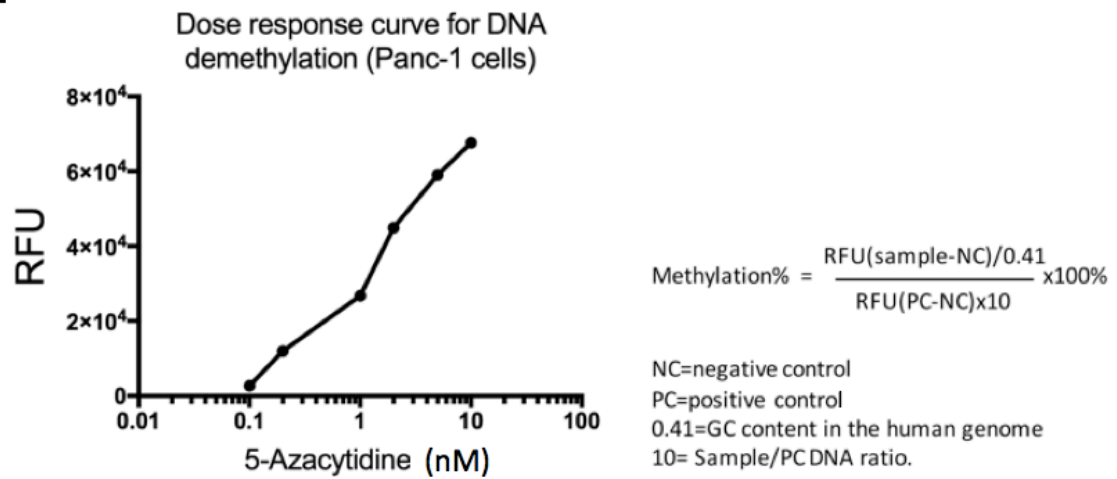
Methylation Percentages:
A0: 4.255774289%
A30: 1.683987775%
A100: 0.918974815%
A300: 0.313439479%

23B

$$\text{MethylDNA (ng)} = \frac{\text{RFU (sample} - \text{NC)}}{\text{slope}}$$

$$\text{Methylation} = \frac{\text{MethylDNA amount}/X}{\text{SampleDNA amount added}} \times 100\%$$

Condition	Methylation %
Cell DNA	4.256%
Cell DNA 5AZA (100nM)	0.919%
Exosome DNA (Untreated Cells)	5.004%
Exosome DNA (5AZA Cells)	1.0008%

Analysis of Exosomal DNA Methylation in PDAC Patients

PDAC Patient Information

In current work, we have begun to evaluate the utility of exosomes for clinical analysis of DNA methylation. We obtained three matched PDAC patient tumor - serum pairs in order to perform a comparison between methylation patterns. This is a significant challenge since only a fraction of exosomes in the patient's circulation originates from the tumor, while other exosomes come from non-cancerous tissues and are likely to have distinct methylation patterns. Thus, it is unclear whether the sensitivity of this method will be sufficient to discern cancer-derived epigenetic signature(s). Patient information is shown in **Table 9**. All patients had a confirmed PDAC diagnosis and underwent a type of tumor resection surgery. We obtained snap-frozen fragments of resected tumor and serum from each patient.

Table 9. Patient Data

Patient	Gender	Procedure	Pathology
PG4	F	Pancreatic Tail Resection	Pancreas pT3N0 (Adeno)
PG6	F	Whipple	Pancreas pT3N1a (Adeno)
PG8	F	Whipple	Pancreas pT3N2 (Adeno)

Differences in Exosome Sequencing Preparation Using Serum Exosomes

DNA was isolated directly from tumor tissue for analysis. For the matched serum samples, a modified exosome isolation procedure via ultracentrifugation was employed using an extended spin time to maximize exosome yield from small-volume

samples. Exosomal DNA yield varied widely between patients, necessitating additional ultracentrifugation of the retained supernatants. Additionally, the DNA isolation protocol was modified, by extending elution procedure from the purification columns for several hours to maximize DNA yields. We elected to perform whole genome bisulfite sequencing (WGBS) instead of RRBS for these samples, due to the lower quality and increased fragmentation of the serum exosome DNA. WGBS does not require site-specific library generation and thus is not hindered by increased fragmentation. A major limitation for methylation-based sequencing analyses is the requirement for sufficient amounts of template DNA for bisulfite conversion prior to sequencing, with large enough fragments for library preparation in case of RRBS. We found that a minimum of 200ng of exosome DNA is required for RRBS/WGBS to be successful, and given the heterogeneity of serum exosomal DNA additional sample is preferable in order to generate sufficient read depth. A schematic of the isolation workflow is presented in **Figure 24**.
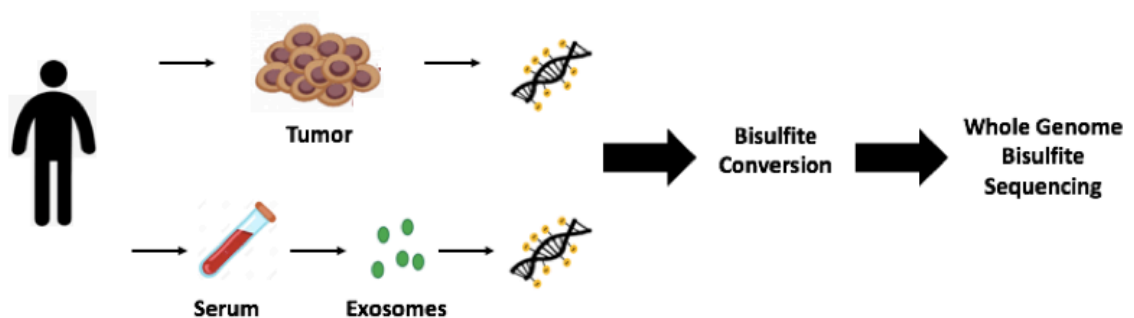
Figure 24. Schematic of patient sample isolation methods.

Currently, we have completed bisulfite conversion, library generation, and sequencing for three matched sets of patient samples (tumor and serum exosome DNA). Obstacles for analysis have included high noise and low base read coverage, likely due to the presence of non-tumor exosomes in the patient serum, a confounding variable not present when isolating exosomes from purified cell line populations. Strategies for optimization may include utilizing a higher volume of input serum for exosome isolation or targeted sequence amplification for relevant site-specific oncogenic methylation changes.

Conclusion

Exosomal DNA is methylated, and as shown above can faithfully recapitulate the methylation patterns of the cell of origin with high fidelity. Additionally, the exosomal DNA methylation marks collectively span the entire genome and can be sequenced and mapped for the analysis of genes of interest. Exosomal DNA methylation patterns can be used to detect site-specific changes in methylation, and can potentially identify oncogenic changes in cancer patients using biological fluid liquid biopsies.

CHAPTER 5

EXOSOME DNA PACKAGING

Introduction

One of the major unanswered questions concerning exosomal DNA is the mechanism of DNA packaging into exosomes. Thus far, while several theories have been put forward, no studies have demonstrated how, or in what contexts, DNA is incorporated into exosomes. It is plausible that the mechanisms enabling the packaging of DNA into exosomes could be cell-type specific and dynamically regulated. While early studies suggested that exosomal DNA may be a characteristic of cancer cells and not of non-tumorigenic cells[47], data presented earlier in this work clearly shows that both tumorigenic and non-tumorigenic lines are capable of producing large amounts of exosomal DNA. The levels of exosomal DNA are however widely variable between different cell lines regardless of tumorigenicity. It has been proposed that exosomal DNA could escape into the cytoplasm at times when the nuclear envelope is compromised or, in the case of mitosis, disassembled[276]. The biogenesis of exosomes involves the formation of multi-vesicular bodies of endosomal origin, which could potentially draw in cytoplasmic components during intraluminal membrane budding, to be encapsulated within exosomes[40]. If DNA, either free or organized in nucleosomes, was present in the cytoplasm, this could be one mechanism by which it could appear in exosomes. Indeed, a recent study by Takahashi et al. proposed that exosomes maintain cellular homeostasis by removing cytosolic DNA from cells, keeping DNA fragments from being sensed by STING and thereby preventing the activation of harmful innate immune responses and reactive oxygen species (ROS)-driven DNA damage responses[108].

The mechanism by which DNA could escape the nucleus, however, remains elusive. A family of proteins have been shown to be frequently disrupted in cancer cells with potential consequences for the integrity of the nuclear envelope. Altered or aberrant expression of the Lamin proteins, specifically Lamins A & C, have been commonly observed to be disrupted in cancer cells, leading to nuclear envelope disruption and other effects that can promote tumor progression[301–303]. Lamins have also been ascribed a role in chromosomal organization and movement during different phases of the cell cycle; this role indicates interactions with DNA[304]. We decided to investigate the hypothesis that exosomal DNA packaging is dependent on nuclear envelope integrity. Here, we sought to determine whether manipulating nuclear envelope integrity by altering Lamin A/C expression could influence exosomal DNA content by increasing the permeability of the nuclear membrane and increasing DNA mobility by reducing lamin-mediated anchorage. This could provide important mechanistic insight for exosomal DNA packaging by linking exosomal DNA content to the structural integrity and permeability of the nucleus.

Linking Nuclear Envelope Integrity and Exosome DNA Content

Demonstration of Lamin A/C Knockdown in Cells

Lamin A/C knockdown has been demonstrated to be non-lethal to cells, due in part to the functional redundancy of Lamin B[305,306]. We performed an siRNA knockdown of Lamin A/C in three cell lines, Panc1, T3M4, and HPNE. The lines were chosen based on their tumorigenicity and exosomal DNA packaging capabilities. Panc1 is tumorigenic, with low baseline DNA content, T3M4 is tumorigenic with high baseline DNA content, and HPNE is non-tumorigenic with very low amounts of

detectable exosomal DNA. Knockdown efficiency in each cell line was validated by qPCR expression analysis (**Figure 25A**).

Exosome DNA Content Correlates With Lamin A/C Expression

After confirming the siRNA knockdown of Lamin A/C in the cells of interest, I proceeded to isolate exosomes from control (untreated, transfection agent alone, scrambled siRNA control), and Lamin A/C knockdown cells. The same number of cells were seeded for each condition and grown to 75-85% confluence prior to exosome collection. DNA was then isolated from exosomes as described previously. For each cell line, Lamin A/C knockdown caused a dramatic increase in exosomal DNA content, which was statistically significant (**Figure 25B**). This work implies a link between exosomal DNA packaging and the integrity of the nuclear envelope.

Conclusion

Here, we demonstrate that by decreasing the expression of the nuclear envelope structural proteins Lamin A & C a corresponding increase in exosomal DNA was observed. This implies that nuclear envelope breakdown or compromise could be an integral part of the process of packaging DNA into exosomes. While further work is necessary to characterize the mechanism by which this occurs, this implicates both normal (cell cycle mediated nuclear envelope breakdown) and pathological (Lamin expression dysregulation) processes in exosomal DNA packaging.

Figure 25. Lamin A/C Knockdown significantly increases exosomal DNA content.

(**A**) Lamin A/C siRNA knockdown validation by qPCR expression assay. (**B**) Exosomal DNA content is significantly upregulated after Lamin A/C knockdown in all three cell lines tested (Panc1, T3M4, and HPNE) when compared with untreated, transfection reagent, and siRNA scramble control conditions. Only one T225 flask of subconfluent cells per condition was used, as compared with the initial exosomal DNA characterization experiments where 3 flasks per line were used.

25A
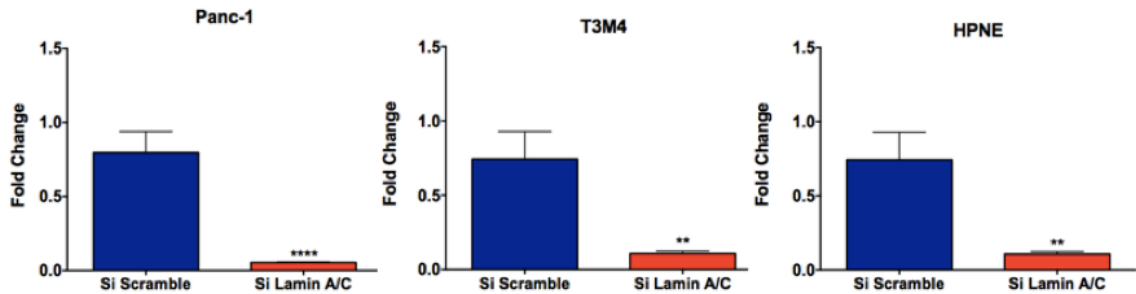
25B
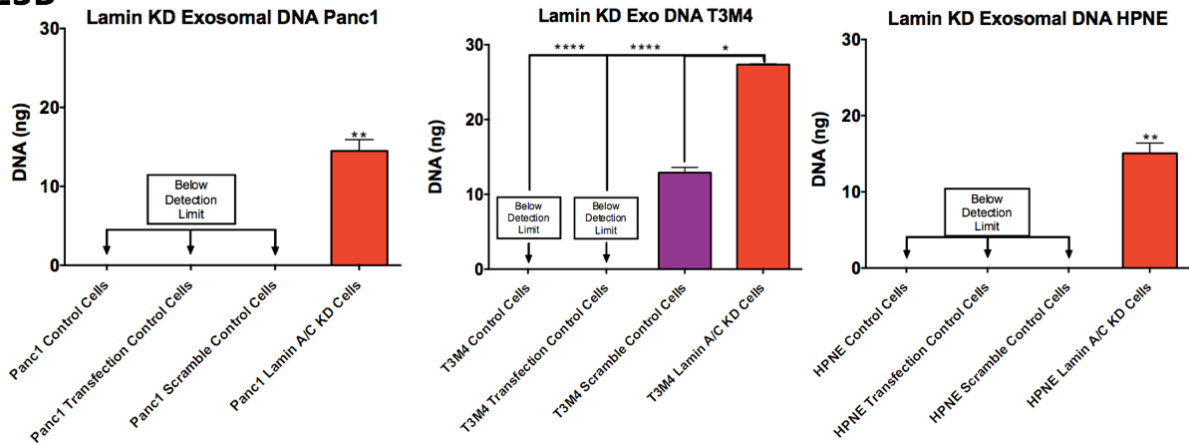
CHAPTER 6

EXOSOMES AS DNA DAMAGE RESPONSE MEDIATORS

Introduction

Increasing evidence suggests that exosomes are critical for cell-cell and cross-tissue communication under both normal physiological conditions and in cancer by shuttling variegated cargo, including DNA, RNA, and proteins, between cells[16,84,202]. Exosome-mediated signaling has been implicated in a variety of functions such as angiogenesis, immunity, proliferation, and tumorigenesis[40,65,152,307]. Since exosomes are released from virtually all cell types, and because their cargo has been shown to vary based on microenvironmental context such as cytotoxic stress, it is likely that their roles are context dependent and are influenced by environmental factors[308].

Chemotherapy and radiation eliminate cancer cells by causing acute genotoxic stress due to massive, genome-wide DNA damage. An unintended consequence of these treatments is the transfer of DNA damage response (DDR) from cancer cells to non-cancerous cells nearby[309]. Termed the bystander effect, this induction of DDR signals has been characterized in cells that have not been directly subjected to DNA damage but exist in proximity to irradiated cells [310,311]. The bystander effect can have deleterious functional consequences, including DNA damage, chromosomal instability, mutations, and apoptosis[309,312]. While some molecular mediators of the bystander effect have been established, including reactive oxygen species (ROS)[313,314], other mechanisms remain unclear. Additionally, almost all studies of the bystander effect focus on radiation-mediated damage, while little is known regarding the bystander effect of soluble damaging agents like chemotherapy. Some studies have suggested soluble factors such as cytokines could play a role[312,315].

Given the well-established roles of exosomes in intercellular signaling, several groups have proposed exosomes as mediators of the bystander DDR effect. Some studies have shown that exosomes derived from irradiated cells show characteristic changes in cargo, including protein and RNA species, that could lead to downstream effects in naïve cells[316–318]. Additionally, it has been demonstrated that exosomes can induce bystander responses in naïve cells, potentially related to the transport of ROS or RNA molecules[319–321]. Overall, however, the mechanisms by which exosomes can generate bystander responses in cells remain vague and undefined. We hypothesize that exosomes mediate paracrine bystander DNA damage responses via the transportation of signaling molecules from cancer cells to naïve recipient cells. Here, we propose a role and a potential mechanism for exosomes in mediating paracrine DNA damage response activation in treatment-naïve, undamaged cells (**Figure 26**).
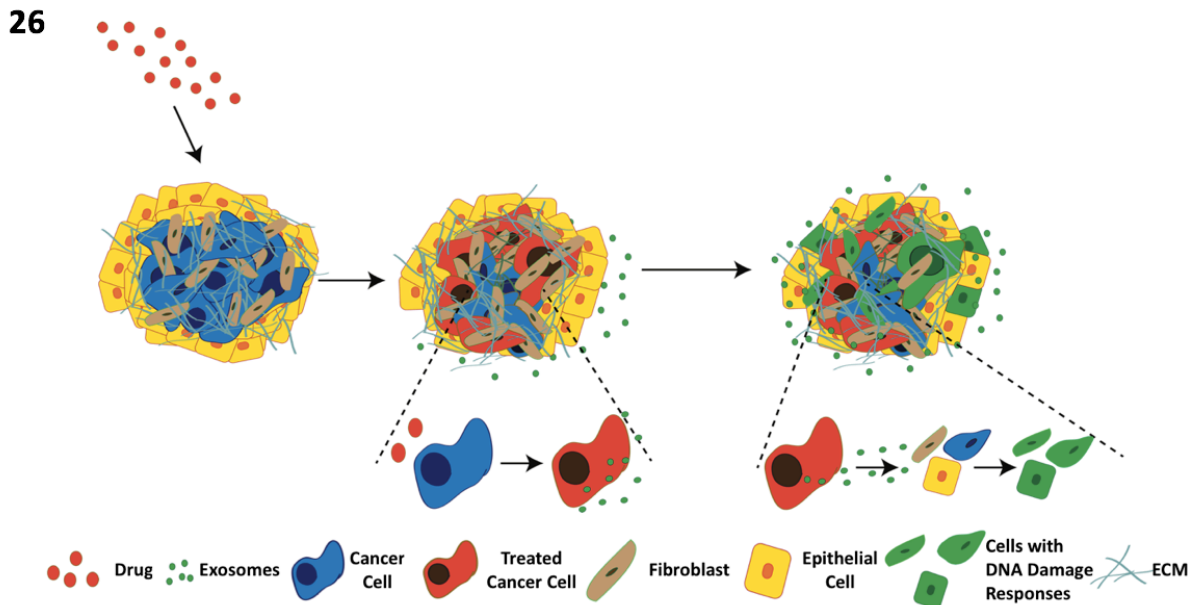
Figure 26. Proposed model of exosome-mediated paracrine DDR activation.

Effects of DNA Damage on Exosomes

DNA Damage Increases Exosome Release

Initially, we decided to characterize some of the properties of exosomes in the context DNA damage using different agents, hydrogen peroxide (H_2O_2) and ultraviolet (UV) radiation. H_2O_2 is a soluble factor that is converted into ROS and generates genome-wide double strand breaks, while UV radiation is a non-soluble DNA-damaging agent that causes the formation of T-T and T-C dimers, subsequently resulting in single and double-strand breaks [322]. We first assessed exosome release in the context of DNA damage in three different PDAC cell lines (Panc1, T3M4, HPNE) and normalized the particle concentration to cell number. We found that while these two DNA-damaging agents did elicit a moderate increase in exosome release across all cell lines, the increase was not significant in comparison with control untreated cells (**Figure 27A**). One caveat to this approach is that the doses of response agents used were optimized specifically to be non-lethal, to enable cell survival for exosome collection, so a dose-response curve may be able to discern whether more toxic, and therefore more stressful, doses of each agent generate more exosomes.

DNA Damage Does Not Impact Exosome Size

We also assessed the average size of the exosomes released by the same three cell lines, under the same conditions. Using Nanoparticle Tracking Analysis (Nanosight) we were able to determine that the size of exosomes is not impacted by DNA damaging agents. In fact, exosomes from each cell line in each condition had remarkably uniform size profiles (**Figure 27B**).

DNA Damage Rarely Impacts Exosome DNA Content

Initially, we hypothesized that DNA damaging agents might generate an increased number of cytoplasmic DNA fragments that would be packaged into exosomes, leading to higher levels of DNA in exosomes released by the cells subjected to DNA damage. In this case, large amounts of fragmented exosomal DNA delivered to recipient cells could be the cause of bystander DDR by activating ectopic DNA sensing molecules in the cytosol, such as STING and Toll-Like Receptors (TLRs)[108,323,324]. However, when we measured DNA in the exosomes released from cells treated with DNA damaging agents, we found very modest upregulation of exosome DNA levels (**Figure 27C**). There was one notable exception; when T3M4 cells were subjected to UV radiation (but not H_2O_2) their exosomal DNA content was increased more than 10-fold compared to all other conditions. This finding again indicates that packaging and sorting of exosomal contents is cell line and context dependent.

Figure 27. Effects of DNA damage on exosome properties.

(**A**) Treatment with either soluble (H_2O_2) or non-soluble (UV) DNA damaging agents modestly increased exosome release in three different cell lines (Panc1, T3M4, HPNE), however none of the differences were statistically significant. (**B**) DNA damage induction did not change the size of exosomes released by cells, as measured by Nanosight NTA. (**C**) DNA damaging agents did not significantly increase exosomal DNA levels, with the exception of UV-damaged T3M4 cells, which showed a dramatic increase in exosomal DNA content after treatment,

27A

Exosomes Released/Cell
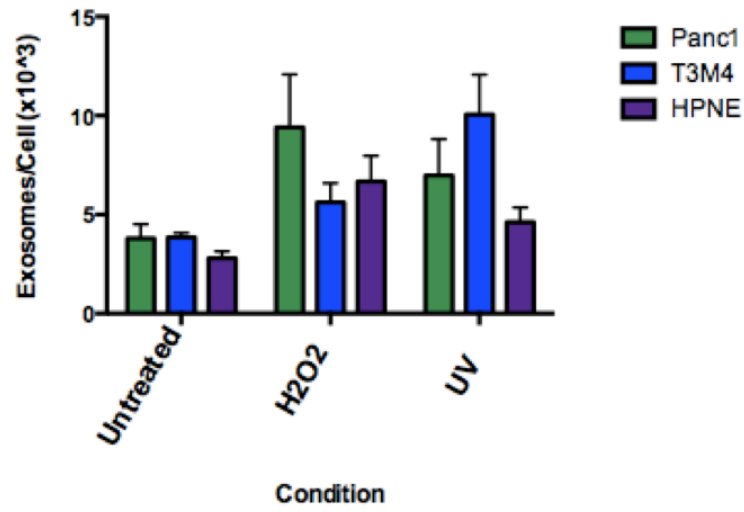
27B

Exosome Size (nm)
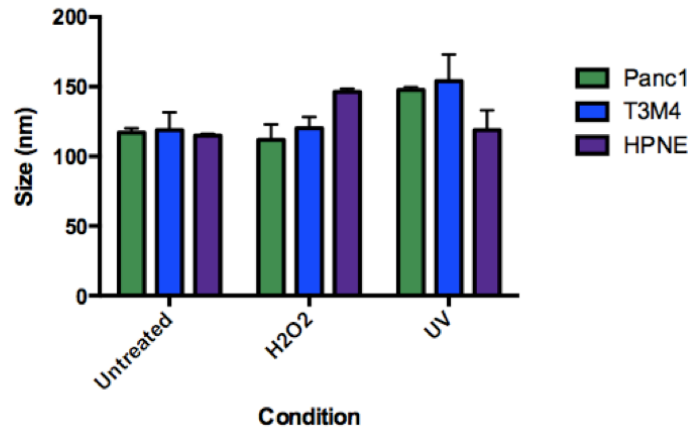
27C

DDR exosomal DNA
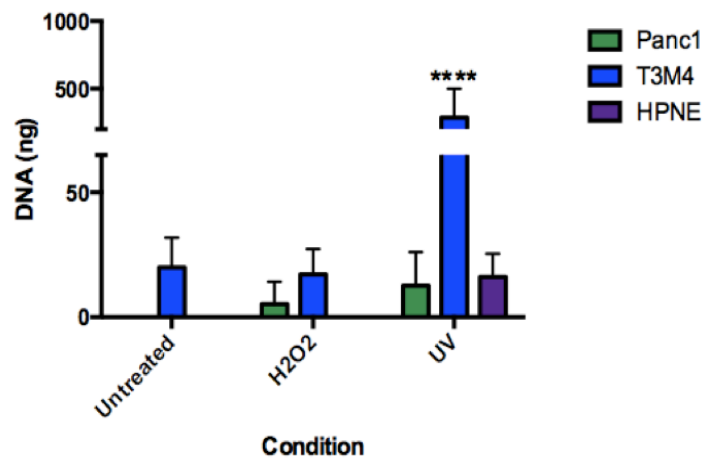
Exosomes from cells treated with DNA damaging agents Elicit Paracrine DNA Damage Responses in Naïve Cells

Exosomes from PDAC Cells with Induced Damage Activate DDR In Naïve Cells.

After establishing that treatment of cells with DNA damaging agents did not appreciably alter exosomal DNA content, with the exception of UV-treated T3M4 cells, we tested exosomes exosomes from identically treated cells for the ability to elicit a DNA damage response in naïve (untreated) cells. We chose to incubate naïve cells with exosomes from the two cancer cell lines, Panc1 and T3M4, that had been exposed either to a soluble (H_2O_2) or non-soluble (UV) DNA damaging agent. We tested four conditions, untreated negative control, exosomes from naïve (untreated) cells, treatment with exosomes from cells following DNA damage, and cells treated directly with the DNA damaging agent of choice (positive control). At the end of treatment, the cells were stained with EdU to visualize actively replicating cells in S-Phase, when homologous repair of DNA double-strand breaks occurs, and p-γH2AX as an indicator of DNA damage response. Nuclei were visualized with the DNA intercalating dye DAPI. We utilized non-malignant pancreatic epithelial cells HPDE as recipient cells, in order to partially recapitulate the balance between cancer cells as donors and normal stroma as recipients of exosomes in the tumor microenvironment. A schematic of the experimental design is shown in **Figure 28**.

Interestingly, when we incubated the treatment-naïve cells with exosomes from the cancer cells exposed to DNA damage, we saw a significant upregulation in p-γH2AX signal, suggestive of DNA damage response. We did not observe a significant
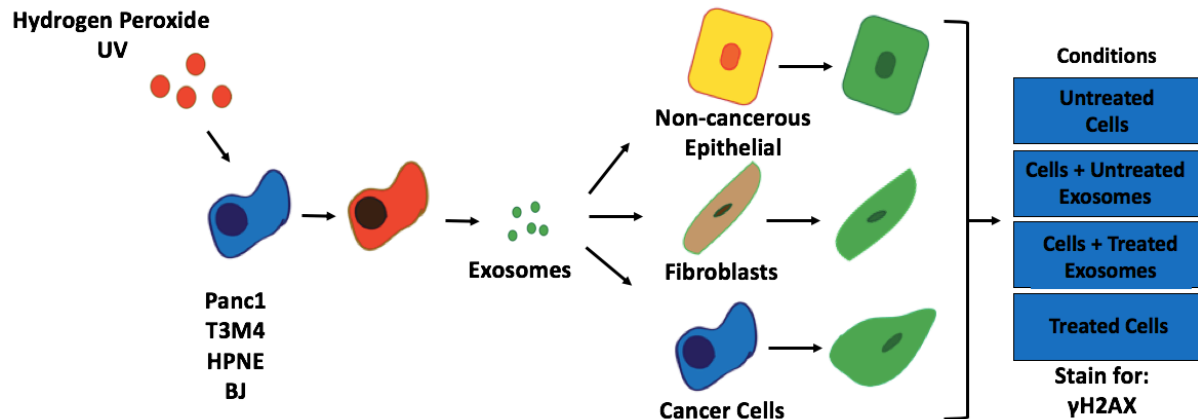
Figure 28. Schematic of DNA damage response experimental design.

Figure 29. Exosomes from damaged cancer cells generate activated DDR in treatment-naïve recipient cells.

(**A**) Staining images of HPDE cells. conditions are untreated negative control, incubated with exosomes from undamaged Panc1 cells, exosomes from H_2O_2-damaged Panc1 cells, and positive control H_2O_2 treatment. DNA damage is indicated by the red p-γH2AX-positive cells, while replicating cells are the green EdU-positive cells. (**B**) Same as A but with T3M4 exosomes. (**C**) Same as A but with UV as the damaging agent. (**D**) Same as B but with UV as the damaging agent. Scale bar 20μm.

29A

	HPDE Cells	HPDE + Panc1 Exo	HPDE + Panc1 H2O2 Exo	HPDE + H2O2
DAPI				
EdU				
p-γH2AX				
Merge				

29B

	HPDE Cells	HPDE + T3M4 Exo	HPDE + T3M4 H2O2 Exo	HPDE + H2O2
DAPI				
EdU				
p-γH2AX				
Merge				

29C

| | HPDE Cells | HPDE + Panc1 Exo | HPDE + Panc1 UV Exo | HPDE + UV |

DAPI

EdU

p-γH2AX

Merge

29D

| | HPDE Cells | HPDE + T3M4 Exo | HPDE + T3M4 UV Exo | HPDE + UV |

DAPI

EdU

p-γH2AX

Merge
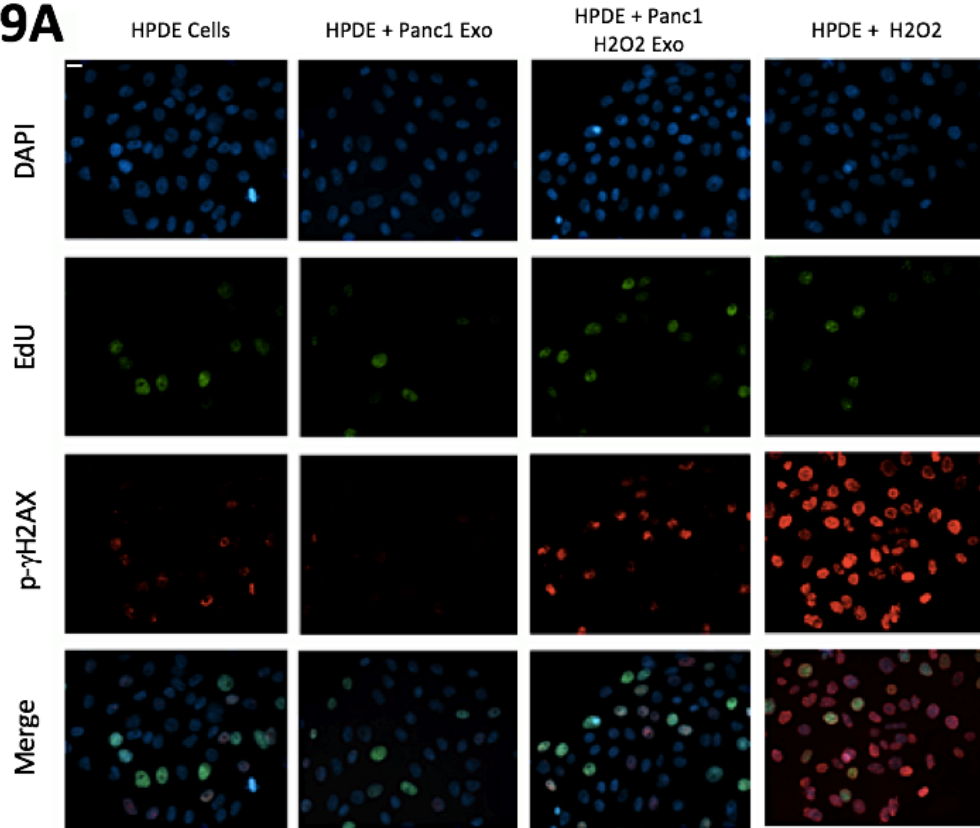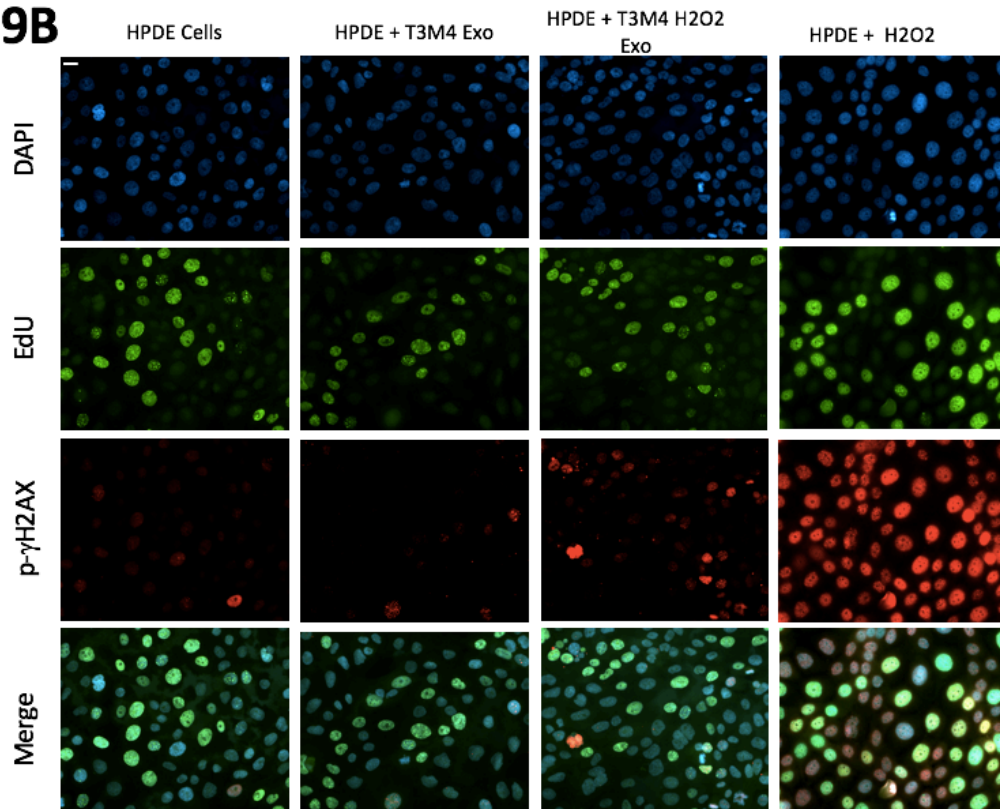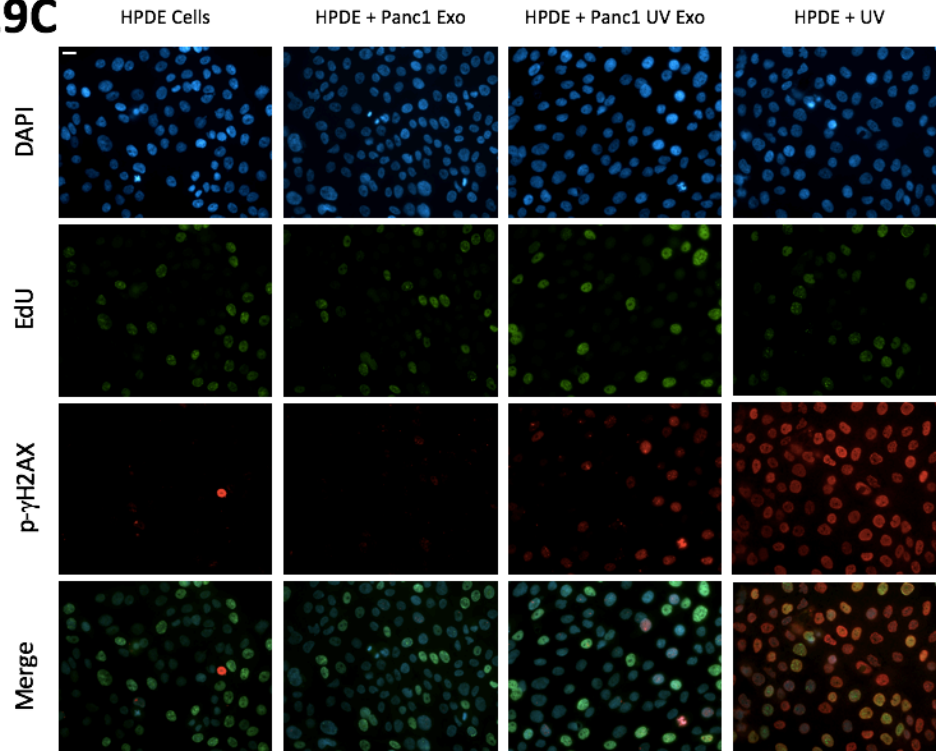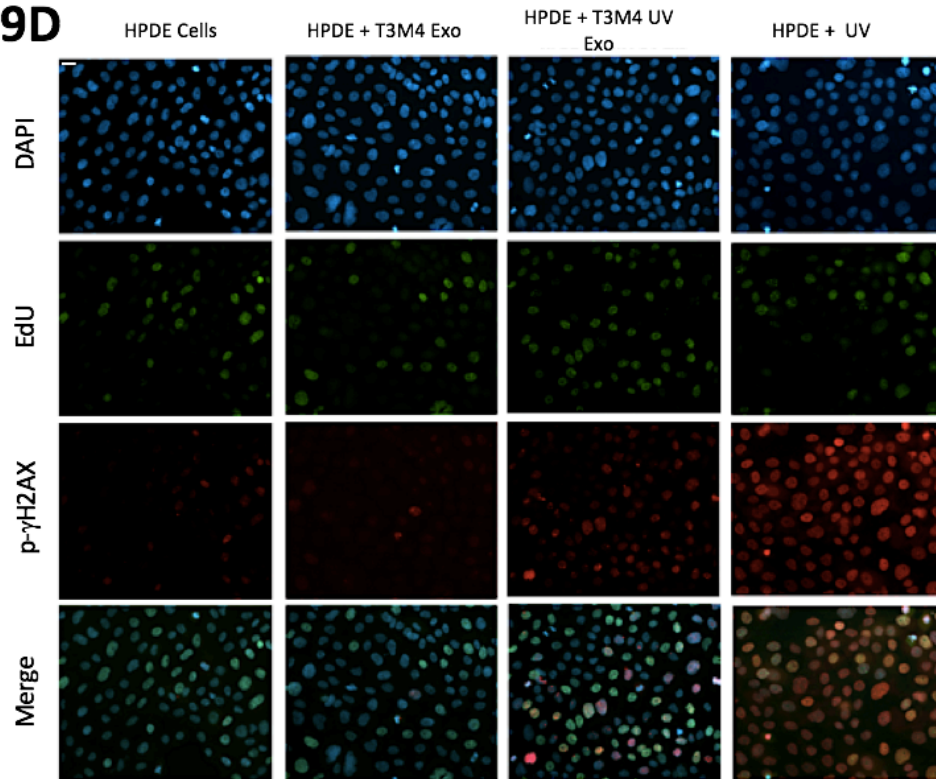
increase in the cells incubated with exosomes from undamaged cancer cells (**Figure 29A-D**). To further analyze this phenomenon, we generated a dose-response by testing increasing concentrations of exosomes for the treatment of naïve cells (2 billion, 10 billion, or 50 billion per condition).We observed a dose-dependent increase in cells positive for p-γH2AX with increasing exosome concentration, demonstrating that the induction of DDR in the recipient cells was exosome mediated (**Figure 30A-D**). The increase in DDR activation signal was observed with exosomes from both Panc1 and T3M4 cells of origin, treated with either H_2O_2 or UV.

Exosomes from Non-Malignant Cell Lines subjected to DNA Damage Do Not Activate DDR in Naïve Cells

Next, we wanted to investigate whether the induction of paracrine DDR activation was specific to exosomes from cancer cells. We induced DNA damage using the UV and H2O2 in two non-malignant cell lines, BJ fibroblasts and HPNE pancreatic epithelial cells. Exosomes were isolated and experiments performed following the same experimental design as above, and we assessed DNA damage activation in the same recipient HPDE cells by percentage of p-γH2AX positive cells. Strikingly, we saw that the exosomes from non-malignant cells failed to induce paracrine DDR in the naïve recipient cells, despite the exposure of the exosome donor cells to DNA damaging agents that were shown to induce DDR (p-γH2AX positivity) in positive control cells (**Figure 31**). This effect was even more apparent when dose response was evaluated and no induction of paracrine DDR was observed with up to 5×10^{10} exosomes (**Figure 32 A-D**).
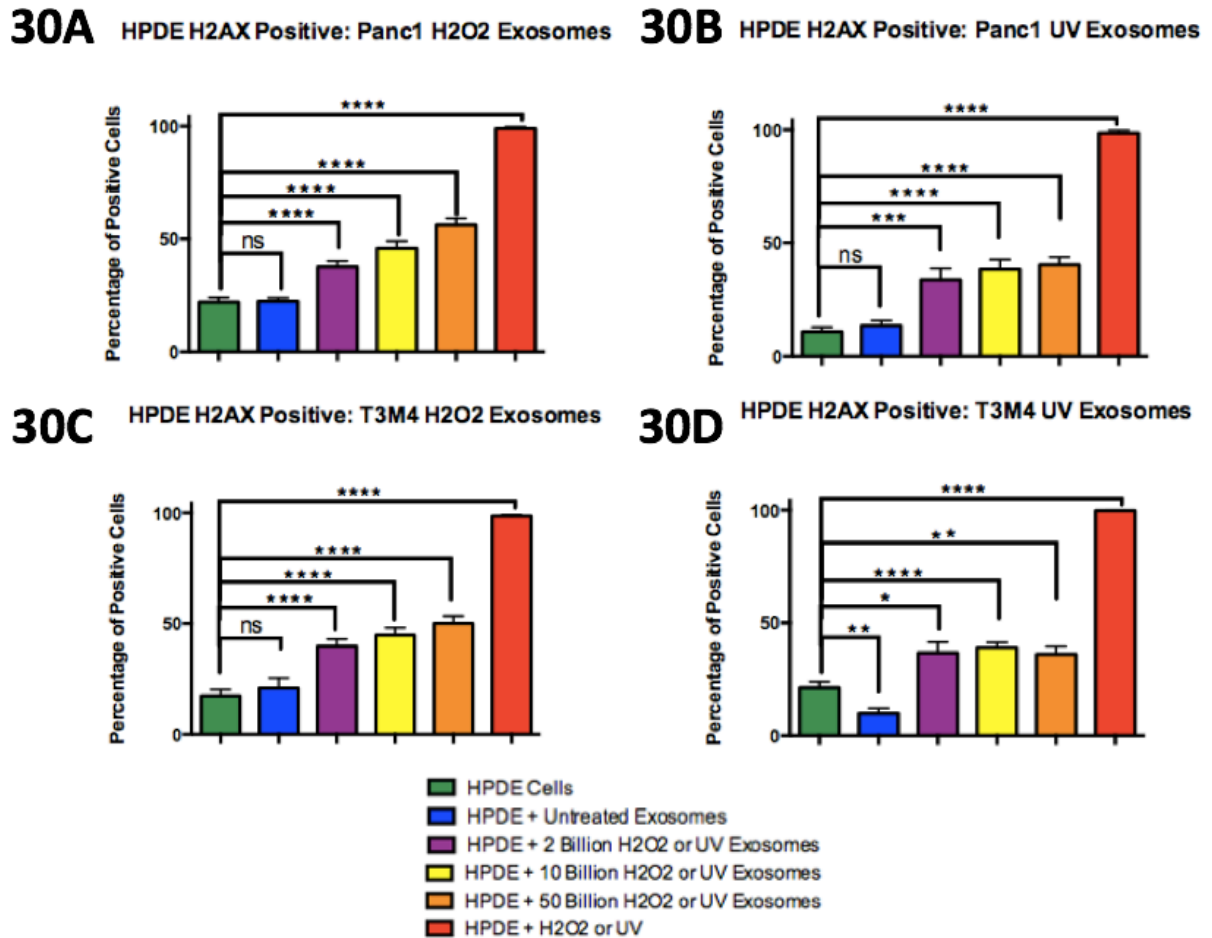
Figure 30. Exosomes from damaged cancer cells increases activation of DDR in recipient cells.

(**A**) Dose response of HPDE cells to exosomes from Panc1 treated with H2O2. Between 2 and 50 billion exosomes were used. Data are expressed as % p-γH2AX positive cells (a marker of DDR activation). There was no significant increase in DDR activation when HPDE cells were incubated with 10 billion exosomes from untreated cells. HPDE cells treated directly with H_2O_2 were used as a positive control. (**B**) Identical experiment was performed using exosomes from Panc-1 cells treated with UV. Positive control – UV-irradiated HPDE cells. (**C**,**D**) Identical experiments were performed using T3M4 cells for exosome collection.

Exosomes from Treatment-Naïve Cells Do Not Induce DDR

We then sought to determine whether exosomes isolated from naïve cancer and non-malignant cells would induce a DDR if applied at higher concentrations. Exosomes were isolated from the same cell lines (Panc1, T3M4, BJ, HPNE), and used to treat HPDE recipient cells at increasing concentrations to assess potential dose response (**Figure 33; 34A-D**). However, we were unable to detect DDR induction upon treatment with exosomes from naïve cells, regardless of exosome number used. These findings suggest that the induction of the paracrine DDR was specific only to exosomes released by cancer cells undergoing genotoxic stress.

Figure 31. Exosomes from non-tumorigenic cells do not induce paracrine DDR activation in recipient cells.

Staining images of HPDE cells. conditions are untreated negative control, incubated with exosomes from undamaged non-tumorigenic HPNE cells, exosomes from H_2O_2-damaged HPNE cells, and positive control H_2O_2 treatment. DNA damage is indicated by the red p-γH2AX-positive cells, while replicating cells are the green EdU-positive cells. No significant increase in DDR was observed in either of the exosome conditions. Scale bar 20μm.
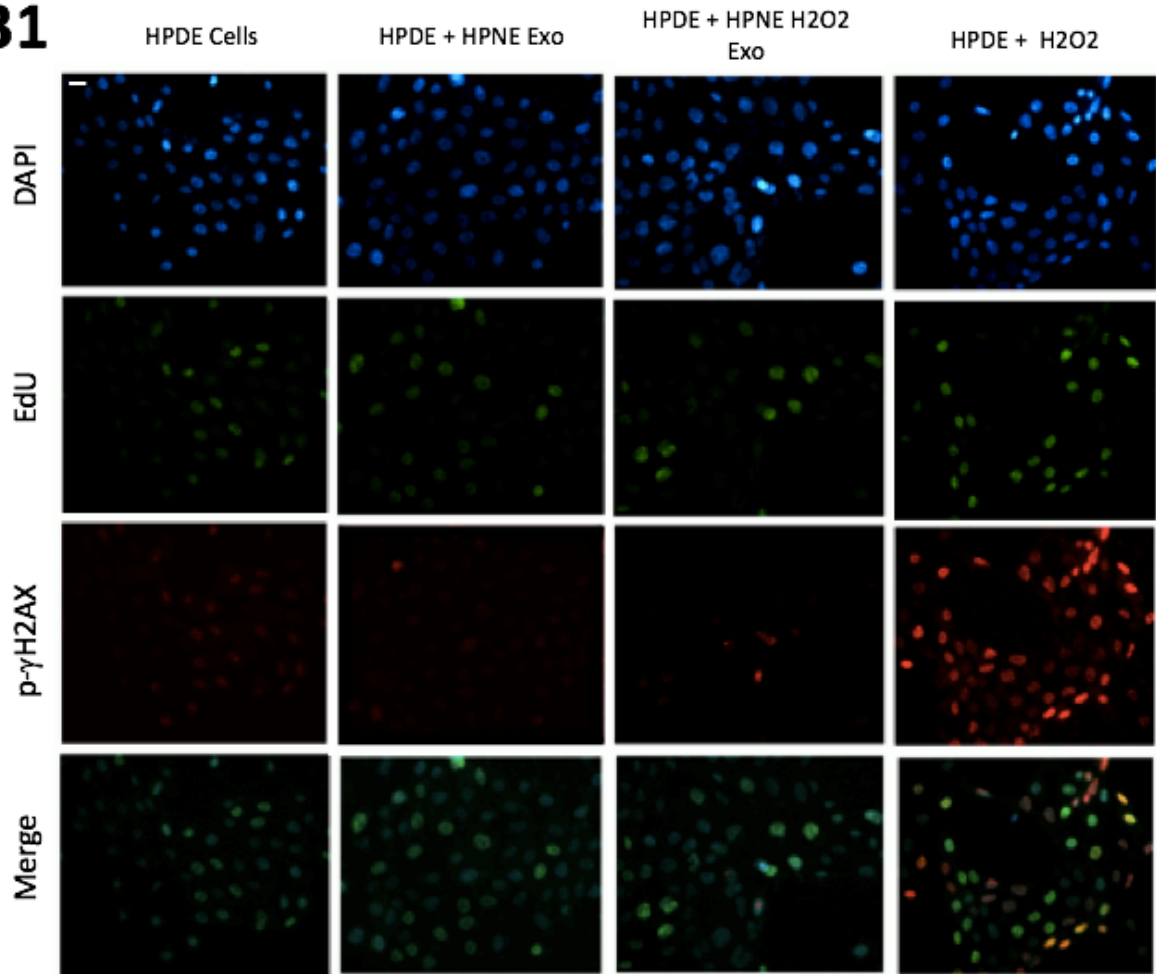
Figure 32. Exosomes from damaged non-tumorigenic cells do not increase activation of DDR in recipient cells.

(**A**) Dose response of HPDE recipient cells to exosomes from H_2O_2-treated HPNE cells, shown as % p-γH2AX positive cells. Note the lack of increase in DDR upon treatment and apparent increase in the positive control (H_2O_2). (**B**) Same as A, with UV as the DNA damaging agent. Positive control, UV-treated HPDE cells. (**C, D**) Experimental design as in A, B using BJ fibroblasts as the exosome donor cells. Scale bar 20μm

32A HPDE H2AX Positive: HPNE H2O2 Exosomes

32B HPDE H2AX Positive: HPNE UV Exosomes

32C HPDE H2AX Positive: BJ H2O2 Exosomes

32D HPDE H2AX Positive: BJ UV Exosomes

Legend:
- HPDE Cells
- HPDE + Untreated Exosomes
- HPDE + 2 Billion H2O2 or UV Exosomes
- HPDE + 10 Billion H2O2 or UV Exosomes
- HPDE + 50 Billion H2O2 or UV Exosomes
- HPDE + H2O2 or UV
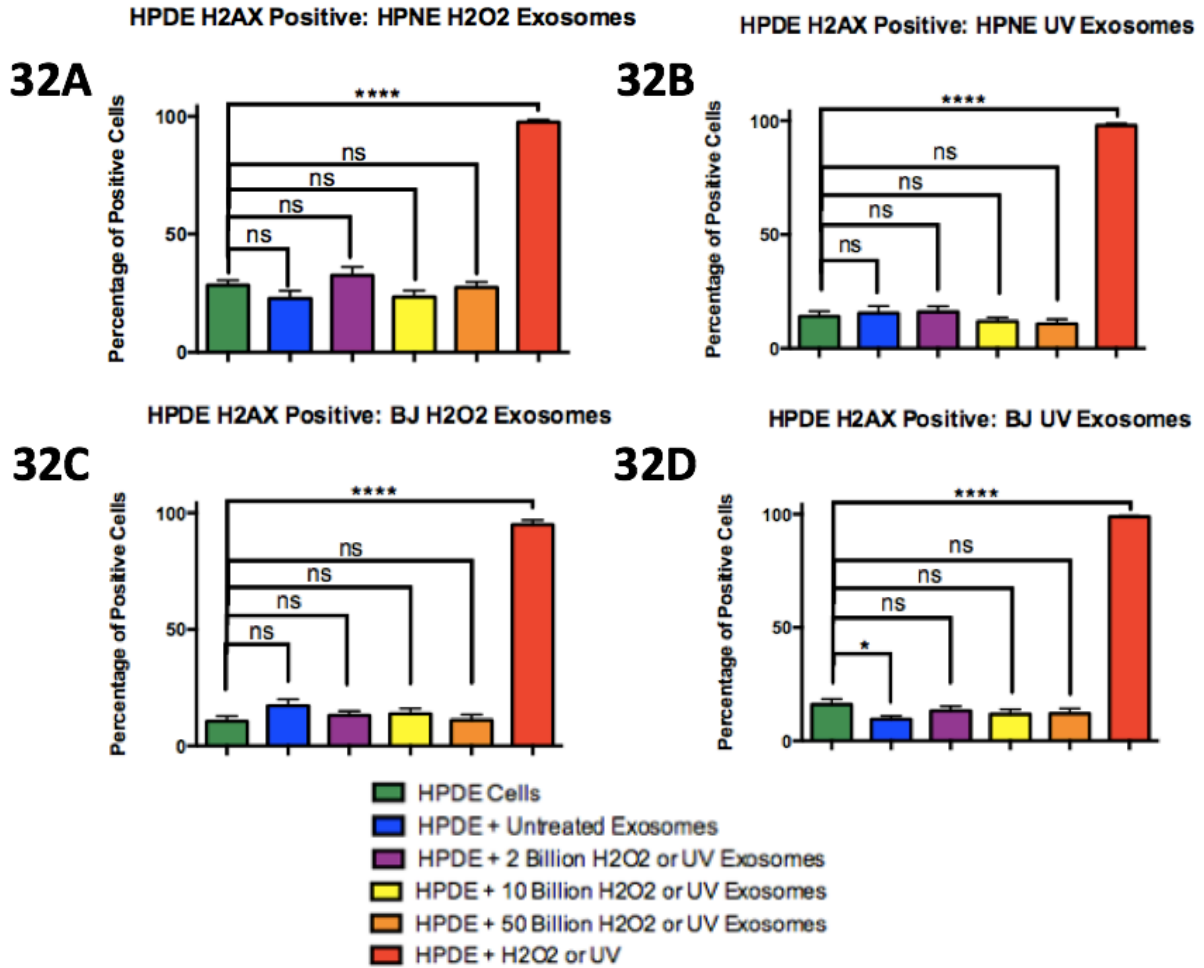
Exosome-induced DDR is independent of Recipient Cell Type

To this point, we had consistently utilized HPDE as the recipient cell line for exosome-induced DDR assessment. To determine if the effect was restricted to HPDE cells, we performed similar experiments using Panc1 cells as exosome recipients, to ascertain whether exosome-induced activated DDR could be elicited in cancer cells as well as non-tumorigenic epithelial cells. Additionally, we wanted to determine whether exosomes could induce a positive feedback loop by propagating DDR in
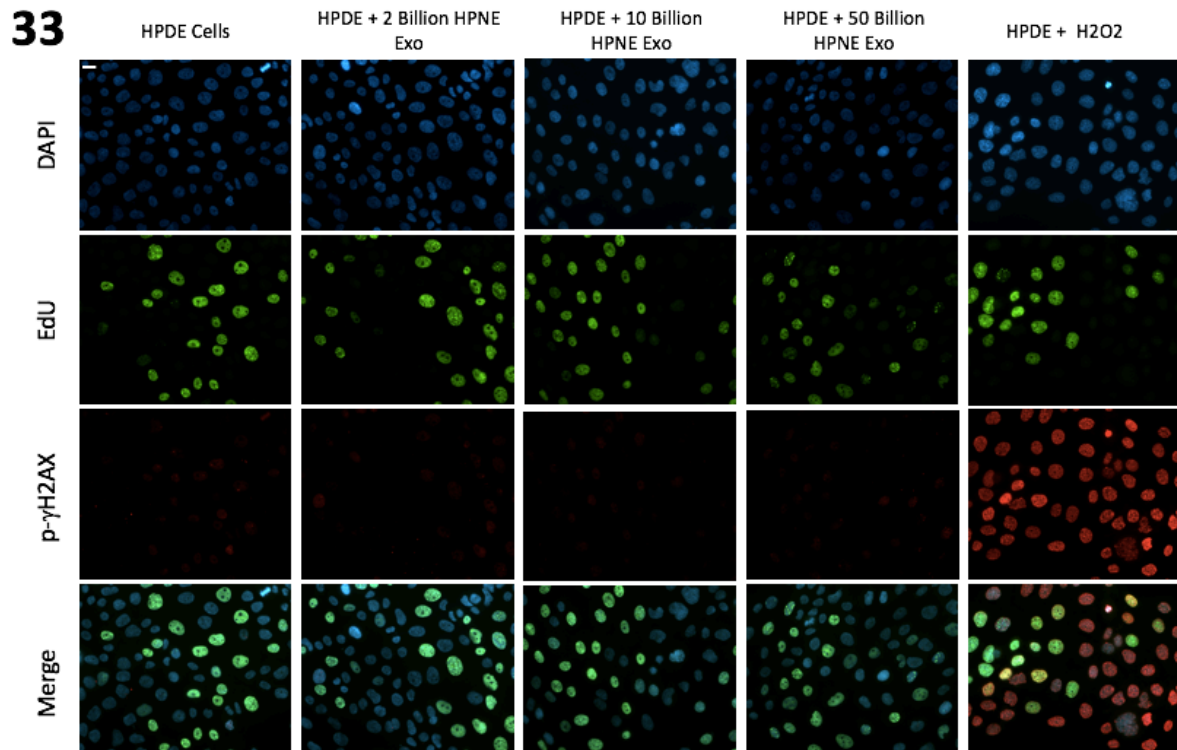
Figure 33. Exosomes from undamaged cells do not induce DDR.

HPDE cells were incubated with increasing concentrations of untreated HPNE cell exosomes. No induction of DNA damage response was observed in the recipient cells regardless of exosome concentration (red signal, p-γH2AX positive cells). Similar results were seen regardless of exosome donor cell line. Scale bar 20μm.

unaffected cells. We repeated the experimental strategies outlined above using naïve untreated Panc1 cells as exosome recipients with nearly identical results, wherein exosomes from cancer cells undergoing DNA damage had the capacity to propagate DDR in the naïve cells, while exosomes from naïve cancer cells and all non-tumorigenic cells post-DNA damage did not induce a significant DDR response (**Figure 35 A-C, 36A-L**).
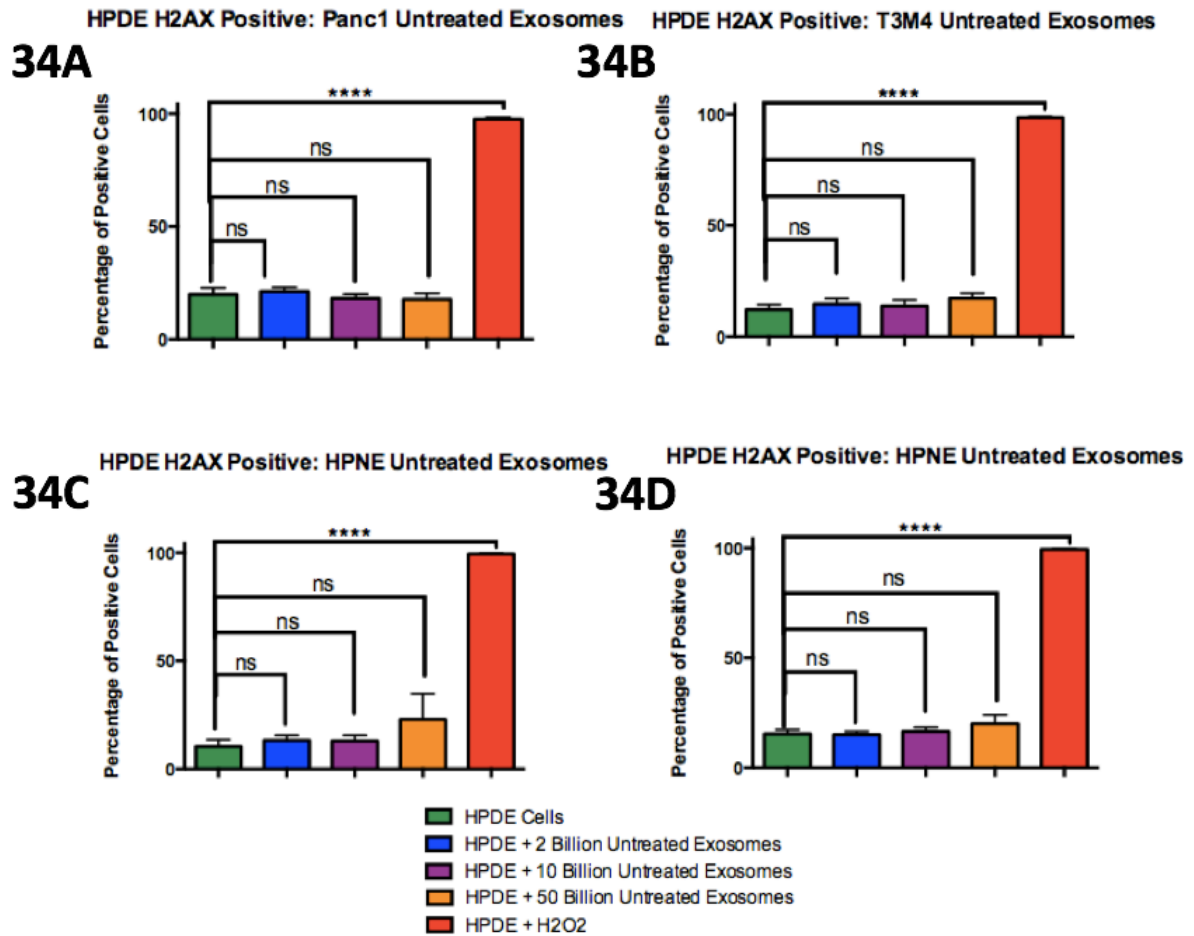
Figure 34. Exosomes from undamaged cells do not increase activation of DDR in recipient cells.

(**A**) Dose response of HPDE recipient cells treated with Panc1 naïve cell exosomes, shown as % p-γH2AX positive cells. There was no significant increase in DDR activation by exosomes from naïve cells, even with high exosome numbers. $H2O2$-treated HPDE cells were used as a positive control. (**B-D**) Identical experimental design was used to test exosomes from T3M4 (B), HPNE (**C**) and BJ cells (**D**), respectively.

35A

| | Panc1 Cells | Panc1 + Panc1 Exo | Panc1 + Panc1 H2O2 Exo | Panc1 + H2O2 |

DAPI

EdU

p-γH2AX

Merge

35B

| | Panc1 Cells | Panc1 + T3M4 Exo | Panc1 + T3M4 UV Exo | Panc1 + UV |

DAPI

EdU

p-γH2AX

Merge
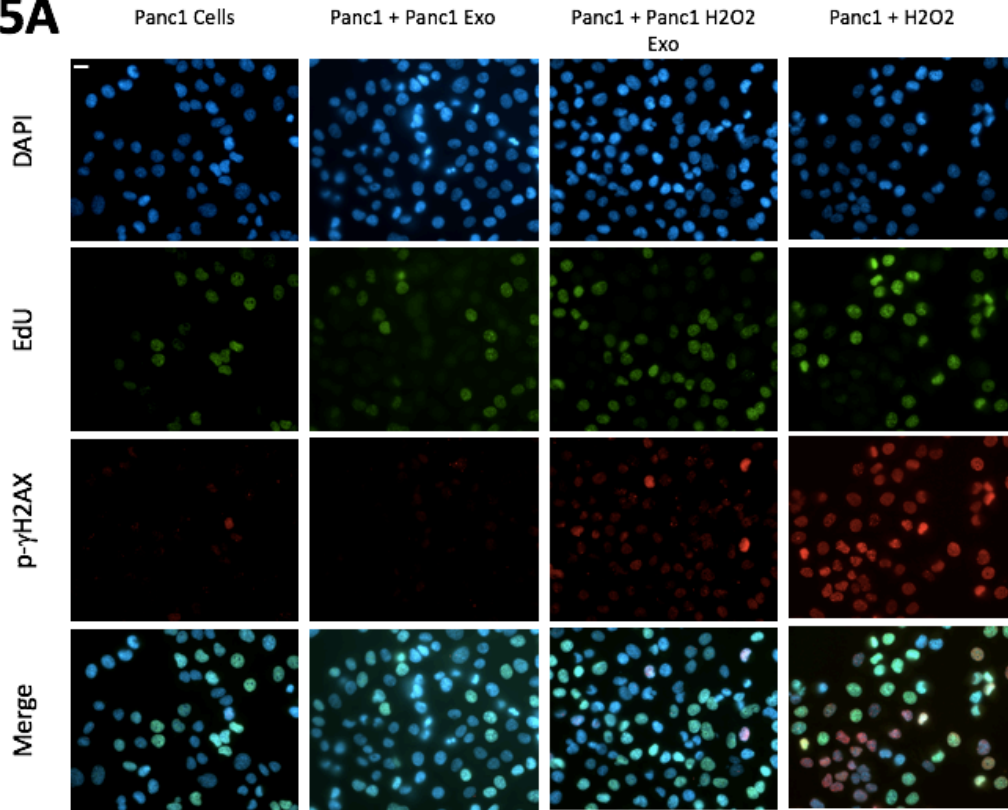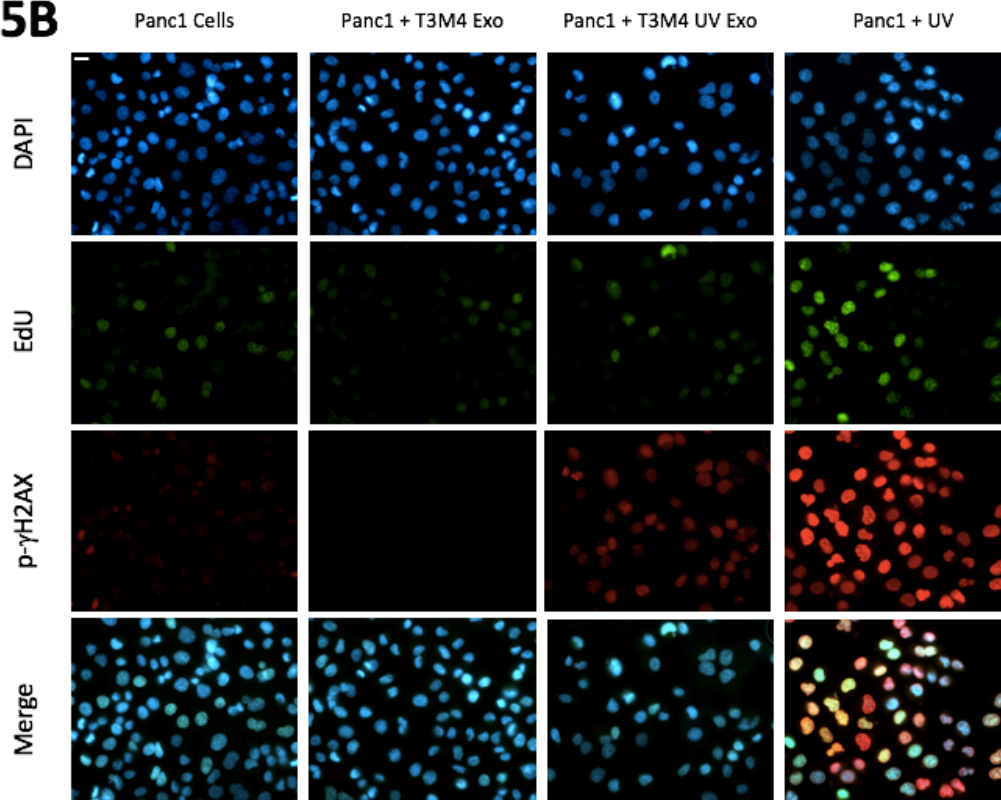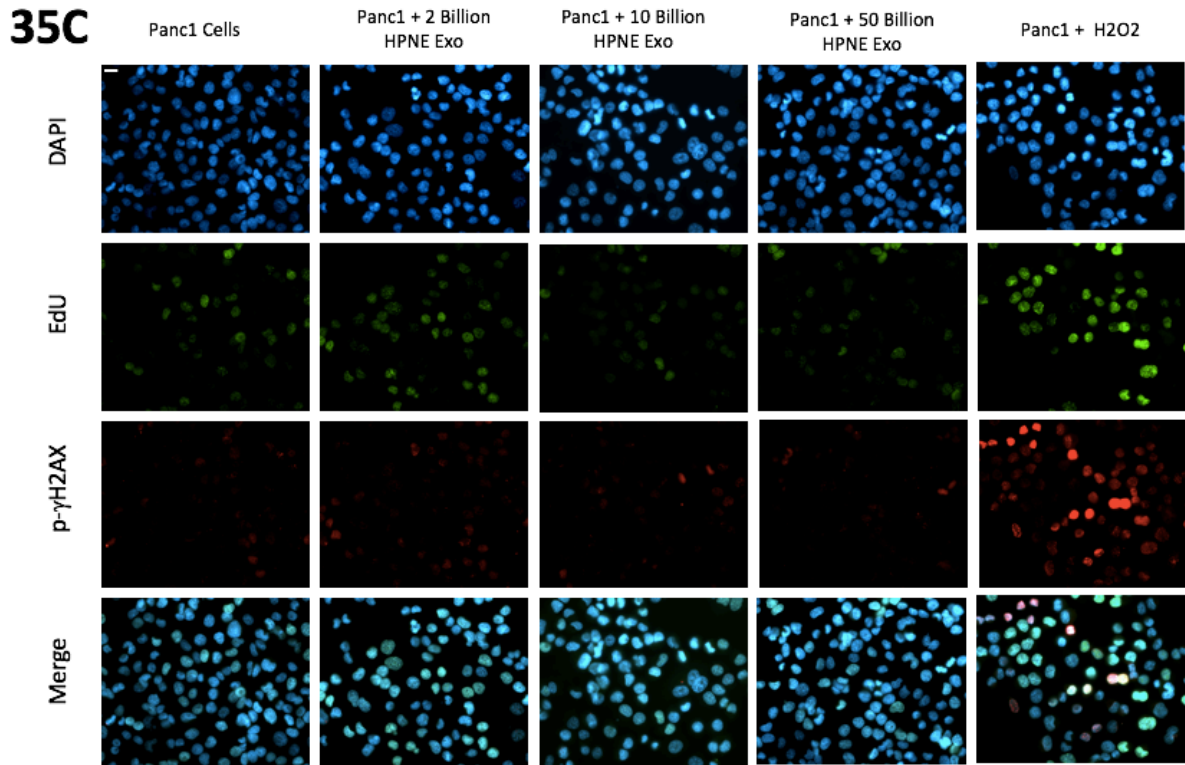
Figure 35 DDR activation experiments done with Panc1 recipient cells.

(**A**) The same experimental strategies for HPDE recipient cells were employed. Consistent with the previous data, exosomes from damaged cancer cells are able to induce DDR in recipient cells. This panel shows the effect of exosomes isolated from Panc1 cells treated with H_2O_2. Interestingly, this also demonstrates that exosomes can induce DDR in cells that are the same type as their cell of origin. (**B**) Same approach as in A, but with T3M4 exosomes and UV as the damaging agent. (**C**) As demonstrated previously, exosomes from undamaged cells still could not generate DDR in recipient cells. Shown are Panc1 cells incubated with exosomes from untreated BJ fibroblasts in increasing concentrations. No significant induction of DDR was observed. Scale bar 20μm.

36A Panc1 H2AX Positive: Panc1 H2O2 Exosomes
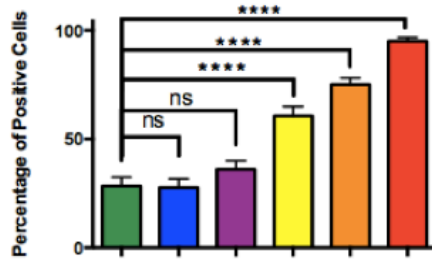
36B Panc1 H2AX Positive: T3M4 H2O2 Exosomes
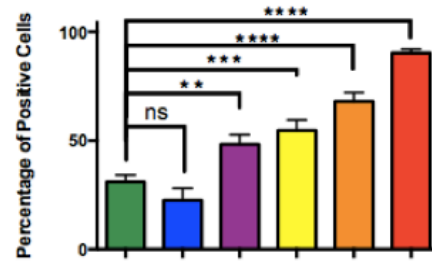
36C Panc1 H2AX Positive: HPNE H2O2 Exosomes
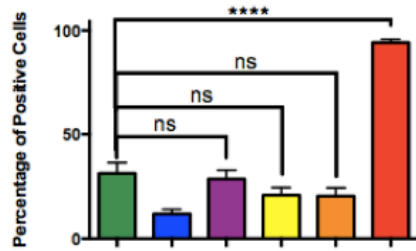
36D Panc1 H2AX Positive: BJ H2O2 Exosomes
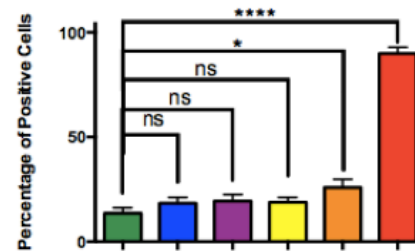
Panc1 Cells
Panc1 + Untreated Exosomes
Panc1 + 2 Billion H2O2 Exosomes
Panc1 + 10 Billion H2O2 Exosomes
Panc1 + 50 Billion H2O2 Exosomes
Panc1 + H2O2

36E Panc1 H2AX Positive: Panc1 UV Exosomes
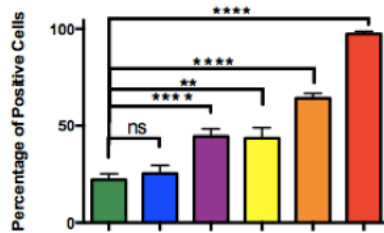
36F Panc1 H2AX Positive: T3M4 UV Exosomes
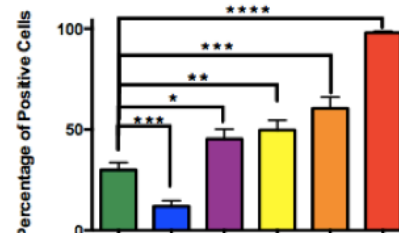
36G Panc1 H2AX Positive: HPNE UV Exosomes
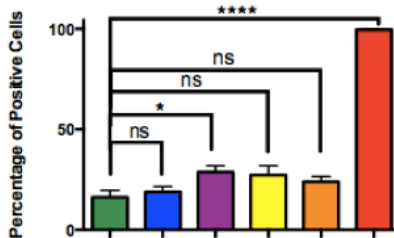
36H Panc1 H2AX Positive: BJ UV Exosomes
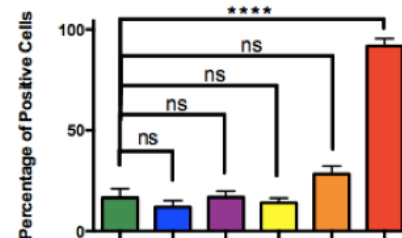
Panc1 Cells
Panc1 + Untreated Exosomes
Panc1 + 2 Billion UV Exosomes
Panc1 + 10 Billion UV Exosomes
Panc1 + 50 Billion UV Exosomes
Panc1 + UV
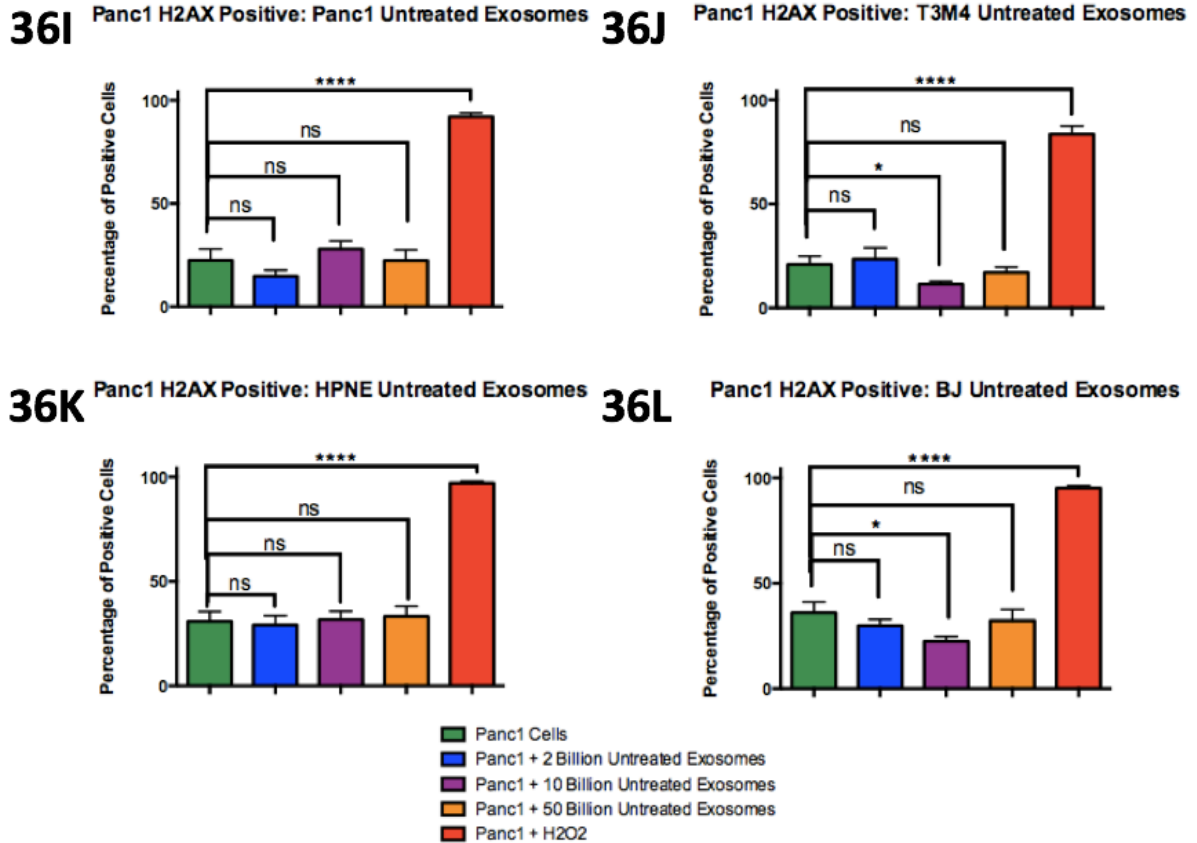
Figure 36. Panc1 recipient cells behave similarly to HPDE recipient cells.

(**A**) Panc1 recipient cells treated with H_2O_2-treated Panc1 exosomes (**B**) Panc1 recipient cells treated with H_2O_2-T3M4 exosomes. (**C**) Panc-1 recipient cells treated with H_2O_2-treated HPNE exosomes (**D**) Panc1 recipient cells treated with H_2O_2-treated BJ exosomes. (**E**) Panc1 recipient cells treated with UV-treated Panc1 exosomes. (**F**) Panc1 recipient cells treated with UV-treated T3M4 exosomes. (**G**) Panc1 recipient cells treated with UV-treated HPNE exosomes. (**H**) Panc1 recipient cells treated with UV-treated BJ exosomes. (**I**) Panc1 recipient cells treated with naive Panc1 exosomes. (**J**) Panc1 recipient cells treated with naive T3M4 exosomes. (**K**) Panc1 recipient cells treated with naive HPNE exosomes. (**L**) Panc1 recipient cells treated with naive BJ exosomes.

Exosome Treatment Does Not Damage the DNA of Recipient Cells

Next, we wanted to determine whether exosomes were somehow causing DNA breaks in the recipient cells, in an effort to determine whether the activated DDR was a result of physical DNA damage in the recipient cells or whether the activation occurred by some other mechanism. To indirectly assess double stranded DNA breaks (DSBs) we performed staining for the DNA repair protein RAD51, which localizes to the physical sites of DNA damage[325]. Thus, in the case of increased DNA damage by exosomes, we expected increased levels of RAD51 in the nuclei of exosome-treated recipient cells. However, we did not observe nuclear RAD51 upregulation in any of the exosome-treated conditions (**Figure 37**). This result implies that recipient cell DNA is not being damaged by exosome treatment, however a major caveat is that RAD51 is involved in homologous repair, and is thus only detectable in S-phase cells. In order to clearly assess the integrity of the recipient cell DNA, additional experiments must be performed, such as Comet assays to assess fragmentation and identify single vs double strand DNA species.

Exosomes From Damaged Cancer Cells Contain Activated DDR Pathway Proteins

To further investigate the potential mechanism by which exosomes from cells undergoing DNA damage propagate DDR, we proceeded to analyze exosome cargo. Since we observed that the exosome DNA content in most cases did not significantly change, it is unlikely that exosome DNA mediates the DDR transmission by exosomes, as was initially hypothesized. We therefore chose to examine exosome protein content, specifically proteins involved in the activation and propagation of DDR.

HPDE RAD51 Positive
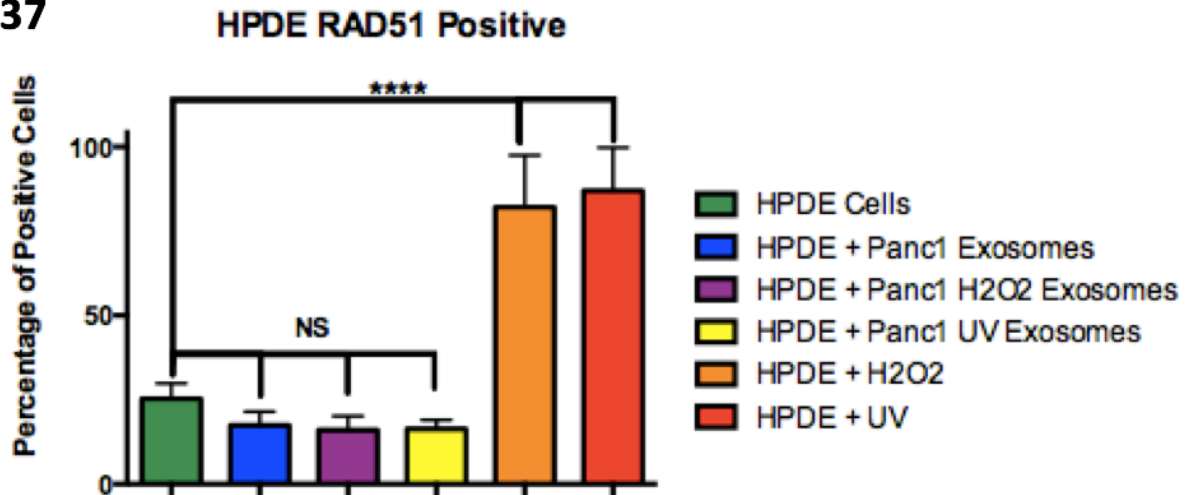
Figure 37. Exosomes do not cause DNA damage in recipient cells.

HPDE recipient cells were incubated with Panc1 exosomes isolated from either naive, H_2O_2, or UV-treated cells. None of these generated an increase in RAD51 expression in recipient cells, suggesting no induction of DNA damage.

There are several proteins involved in the sensing of DNA damage, as well as subsequent downstream activation of DDR mediators and effectors that dictate cell fate outcomes. We posited that, potentially, exosomes might be transferring activated DDR pathway proteins to recipient cells, allowing for horizontal activation in the recipient cells. We focused specifically on the proteins CHK1 and CHK2 downstream of the apical kinases ATR and ATM, respectively, in the DNA damage response pathway[325].

CHK1 and CHK2 are 56 and 62 kDa proteins that could be easily packaged into exosomes. In the canonical DDR pathway, CHK1 and CHK2 are phosphorylated by ATR/ATM and recruit downstream effectors such as p53 and CDC25[325]. We

generated protein lysates from three cell lines (Panc1, T3M4, HPNE) under 3 different conditions (naïve, H_2O_2-treated, UV-treated), and performed Western blots for phosphorylated CHK2. Phospho-CHK2 was indeed present and upregulated in cells when DNA damage was induced (**Figure 38A**). Next, we probed for p-CHK2 in protein extracts from exosomes from the same cells, identically treated. We observed that, highly consistent with the results of p-γH2AX staining, p-CHK2 was increased in the exosomes isolated from cells that underwent DNA damage, and was much higher in the exosomes from cancer cells compared to exosomes from non-malignant cells exposed to the DNA damaging agents (**Figure 38B**). Finally, we probed for p-CHK1, with similar results. Interestingly, however, p-CHK1 levels were relatively high in exosomes from naïve cancer cells (**Figure 38C**).

Conclusion

This exciting data is the first reported evidence that exosomes carry activated DNA damage response proteins that could possibly have functional outcomes in recipient cells. While further analysis is required to better establish and characterize the shuttling of these proteins to recipient cells via exosomes, this is promising evidence of a potential mechanism of exosome-mediated paracrine DDR activation. A schematic of the proposed mechanism is shown in **Figure 39**. Interestingly, only exosomes from damaged malignant cells were able to induce DDR in recipient cells, which could be due to survival adaptations that are tumor specific. While further investigation is required to elucidate the exact mechanisms of exosome-mediated DDR activation, this data provides evidence that exosomes are important signaling vehicles in the tumor microenvironment.

38A

P-CHK2 Cellular Protein

Panc1   T3M4   HPNE

62 KDa →

| H$_2$O$_2$ | − | + | − | − | + | − | − | + | − |
| UV | − | − | + | − | − | + | − | − | + |

β-Actin
Loading Control
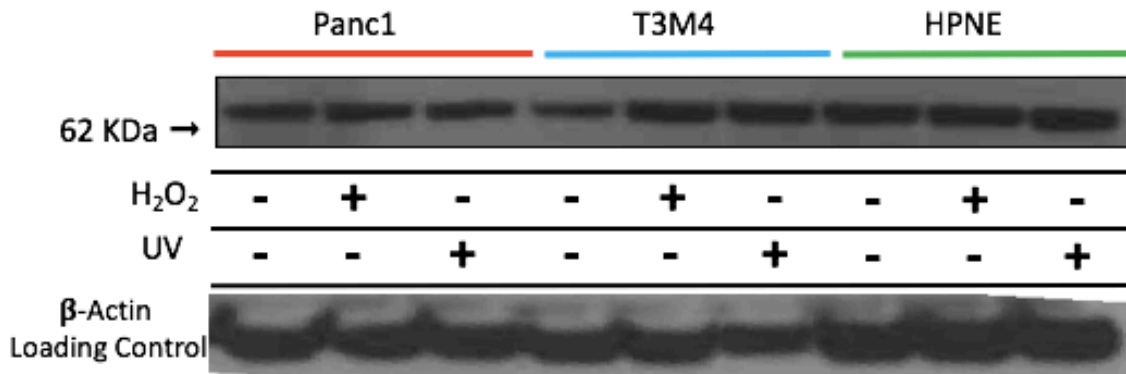
38B

P-CHK2 Exosome Protein

Panc1   T3M4   HPNE

62 KDa →

| H$_2$O$_2$ | − | + | − | − | + | − | − | + | − |
| UV | − | − | + | − | − | + | − | − | + |

β-Actin
Loading Control
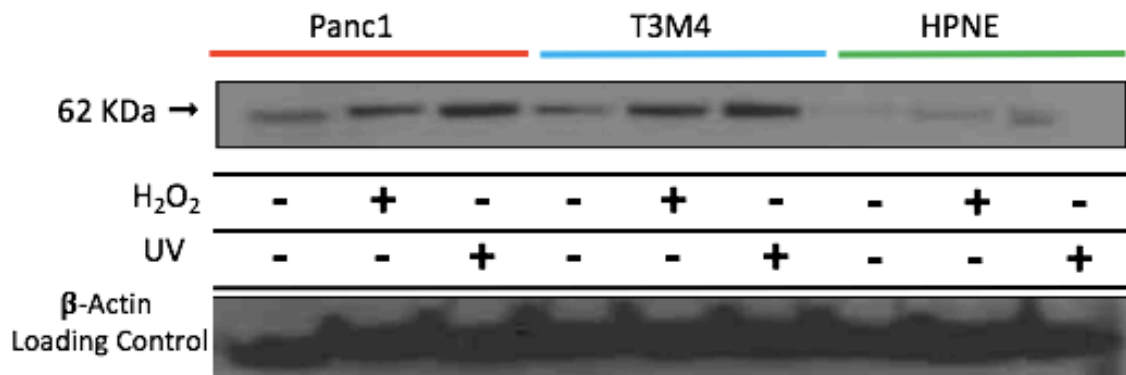
38C

P-CHK1 Exosome Protein

Panc1   T3M4   HPNE

56 KDa →

| H$_2$O$_2$ | − | + | − | − | + | − | − | + | − |
| UV | − | − | + | − | − | + | − | − | + |

β-Actin
Loading Control
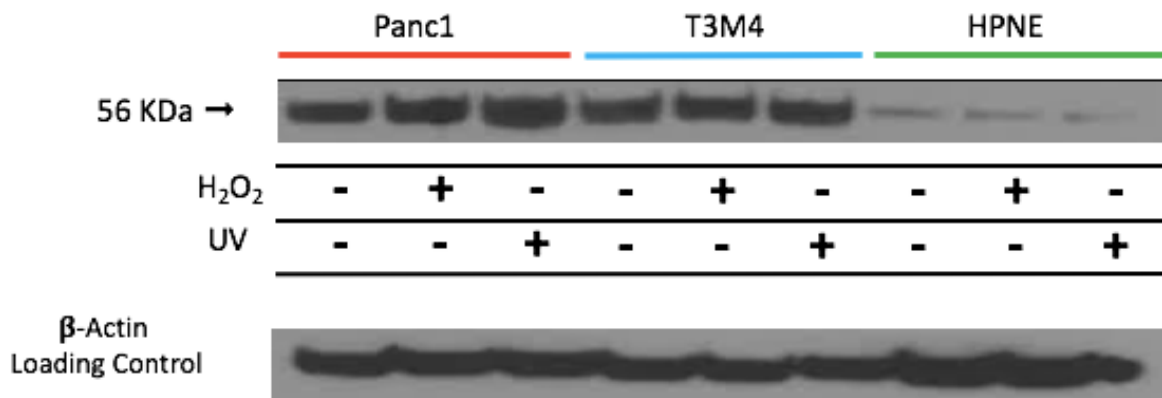
Figure 38. Western Blots for p-CHK1 and p-CHK2 show the presence of activated DDR proteins in cancer cell exosomes with induced DNA damage.

(**A**) Protein lysates from Panc1, T3M4, and HPNE cell lines untreated, H_2O_2 treated, or UV treated were indicated were run on polyacrylamide gels, transferred to PVDF membranes and proved with antibodies for phospho-CHK2. Phosphorylated CHK2 is present in cells and is upregulated upon DNA damage induction, as expected. (**B**) Blot for p-CHK2 using protein lysates isolated from the same cells treated with indicated agents, as in A. Phosphorylated CHK2 is present in exosomes and increased in those isolated from cancer cells undergoing DNA damage. (**C**) Blot for p-CHK1 using the same exosome lysates as in B. Phosphorylated CHK1 is also present in exosomes and increased in those isolated from damaged cancer cells. Interestingly, p-CHK1 was also highly expressed in undamaged cancer cell exosomes.

39
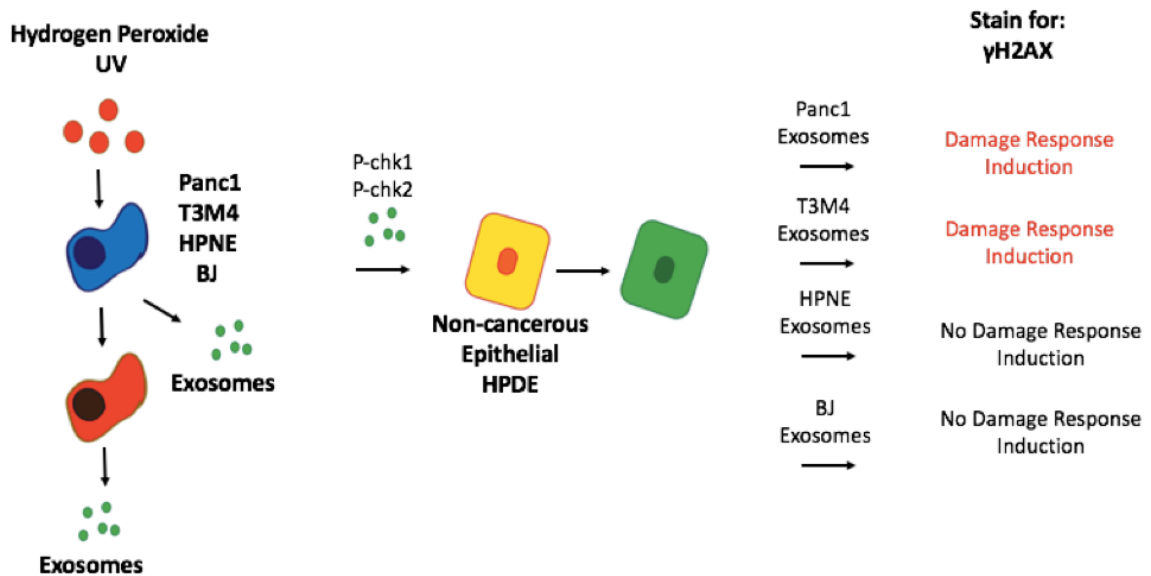
Figure 39. **Working Model of Exosome DDR Activation.**

CHAPTER 7

DISCUSSION AND FUTURE DIRECTIONS

Discussion

Characterization of Exosomal DNA

Exosomes have been repeatedly demonstrated to have heterogeneous functions and cargo, implying potential roles for them in a variety of biological contexts[202]. While many of the molecules found in exosomes, such as proteins and RNA, have been extensively investigated, exosomal DNA has remained largely unexplored. Here, we sought to provide better characterization of exosomal DNA and establish groundwork for further study.

The canonical definition of exosomes is that they are heterogeneous vesicles surrounded by lipid bilayer, between 50-150nm in size, released from multi-vesicular bodies into the extracellular milieu, and eventually make their way into body fluids including blood, urine, saliva, etc[40]. True to the definition of heterogeneity, exosomes are broadly varied in terms of their DNA content, depending on their cell line of origin. Through the isolation of exosomal DNA from a large number of cell lines spanning a wide variety of malignant and non-malignant cell types, we were able to show that the amount of DNA packaged into exosomes changes on a cell-by-cell basis. This is an important consideration for the potential development of diagnostic tools based on exosomal DNA, as their potential utility would depend on how much DNA the cells /tissues of interest, such as tumors, package into their exosomes.

Evaluation of Exosome Isolation Methods for DNA Collection

Among the most controversial topics in the exosome field is identifying the best method of exosome isolation. As exosomes have grown in popularity in the research community, numerous isolation technologies, from affinity columns to precipitation

reagents, have been developed. Each method has distinct advantages and drawbacks, and ultimately the best technique likely depends on the desired downstream application. In the context of exosomal DNA, a recent study by Jeppesen et al[218] utilized the method of ultracentrifugation in a buoyancy flotation iodixanol gradient (IG), yielding high purity exosome populations based on particle size and surface markers. However, these particles were devoid of DNA. We chose to investigate this method, as well as the two other most commonly used isolation techniques, size exclusion chromatography (SEC) and ultracentrifugation (UC), to provide a comprehensive evaluation of exosomal DNA and the effects of different isolation methods on the DNA yields and quality. We were able to definitively prove the presence of double-stranded DNA content associated with exosomes isolated by each method, including IG. Additionally, we utilized DNase I treatment prior to exosome lysis to demonstrate that intravesicular DNA content is protected from DNase digestion by the lipid bilayer. Therefore, the DNase resistant DNA associated with exosomes is localized inside the exosome lumen. We employed multiple forms of characterization, including flow cytometry, western blotting for established exosome markers, and nanoparticle tracking analysis (NTA) to demonstrate the purity, integrity and identity of the exosomes.

Overall, while we showed that exosomal DNA is present in exosomes isolated by each method, not every method is was equally efficient in terms of the quality and quantity of exosomal DNA extracted. UC generated the largest amount of total DNA, however it was most susceptible to DNase treatment, showing that much of the DNA in UC exosome preparations is extravesicular. There were also other contaminants

present, such as cell-free DNA and protein. This is to be expected, as UC isolation is based on sedimentation by centrifugation forces and precipitates all material in a given sample. The optiPrep density gradient is designed to separate subfractions of exosomes by size, and indeed with the high number of ultracentrifugation washes involved this method yielded relatively pure populations. However, due to the multiple wash steps, the yields were surprisingly low, despite high input (number of flasks/cells used for exosome harvest). The quality of the DNA, as ascertained by chip electrophoresis, was generally much lower as well. It has been shown that high-speed ultracentrifugation can be damaging to exosome membranes[235], and while the optiPrep itself may be a gentle mode of isolation, the four wash steps could serve to rupture and degrade exosomes and their contents, making this method less than ideal in terms of the yields of exosomes and luminal cargo for further applications. The best method for the isolation of exosomal DNA, at least among those tested by us, was size exclusion chromatography. Known to be a kinder method of obtaining exosomes, as it uses the force of gravity to pass samples through a gel-filtration resin, we saw the highest yields with the least susceptibility to DNase treatment. This coupled with the ease of isolation and minimal exosome loss pointed to SEC as the superior technique for collecting exosomal DNA.

Exosomal DNA is Methylated

Only two groups have previously shown the presence of methylation marks on exosomal DNA[47,289]. Additionally, neither went into much depth in terms of characterizing the scale of exosomal DNA methylation or testing whether exosome methylation patterns could readily and reproducibly provide insight into the epigenome

of the cells of origin, such as pro-tumorigenic methylation states. Here, we demonstrated conclusively that exosomal DNA is, in fact, methylated. We were able to successfully perform reduced representation bisulfite sequencing and characterize the methylome of both tumorigenic and non-tumorigenic human pancreatic cells. Furthermore, with only ~200ng of input DNA, we were able to evaluate each sample on an individual base-pair level. This allowed an investigation into site-specific promoter methylation patterns, which led us to identify matched differentially methylated sites in the exosomal and nuclear DNA from tumorigenic vs. non-tumorigenic cells. This opens up the potential opportunity to utilize exosomal DNA methylation patterns as a complement to conventional mutation analysis for cancer diagnostics and monitoring. Since exosomes are readily isolated in the bodily fluids of patients, exosomal DNA mutations and methylation patterns could be utilized as a viable non-invasive method for liquid biopsy.

Exosomal DNA Methylation Patterns Are Cell Line Specific

Using correlation analyses, we compared the methylation patterns obtained from exosomes isolated from different cell lines. Remarkably, the exosomal DNA not only almost fully recapitulated the methylation patterns of the cells of origin, but it could also be used to directly identify the cells of origin. Therefore, we indicate for the first time that exosomal DNA carries a distinct, cell-specific signature that can be readily detected for analysis and evaluation. This characteristic could prove invaluable for the early detection of diseases like cancer, which are often asymptomatic and difficult to detect in early stages. In preliminary work, we isolated DNA from matched tumor tissue and serum exosomes from PDAC patients for initial evaluation of the clinical

utility of exosomal DNA methylation for tumor detection. Thus far, we have been able to successfully perform whole genome methylation sequencing of the serum exosome DNA, and sequence analysis is ongoing.

Exosome DNA Packaging

To date, there is no established mechanism for the packaging of DNA into exosomes. While several studies have suggested that factors such as increased DNA damage and fragmentation, as well as the genomic instability inherent to diseases such as cancer, could lead to more DNA becoming available to be sorted into exosomes[276], no work has been done to specifically identify how DNA could escape the nucleus into the cytosol for subsequent incorporation into exosomes for extracellular release. Here, our initial findings indicate the potential importance of nuclear membrane integrity for the loading of exosomes into DNA. Upon successful knockdown of Lamins A and C, two major structural components of nuclear envelope, we observed a significant increase in exosomal DNA in three cell lines with different phenotypes.

Thus, while further investigation is still needed, we propose that the disruption of nuclear envelope is a critical part of the mechanism of DNA transfer from the nucleus to multi-vesicular bodies. One of the major unanswered questions regarding DNA packaging into exosomes is how exosome DNA, cumulatively, can span the whole genome with no apparent biases. If nuclear envelope integrity is in fact critical for exosomal DNA packaging, then the nuclear breakdown during cell cycle progression may be the next critical area of investigation regarding the mechanism of DNA packaging into exosomes. An interesting potential experiment would involve cell

cycle arrest to synchronize cells, releasing them into S phase, and then damaging the DNA in a site-specific manner, using an agent such as cisplatin, to observe whether a parallel increase in site-specific DNA fragments are found in exosomes isolated from those cells. This would provide crucial information regarding the mechanism of exosomal DNA packaging and its potential relationship to nuclear envelope integrity, cell cycle progression, and DNA damage. Understanding the mechanism of exosomal DNA packaging would provide critical insight that could be used to identify the best contexts in which exosomal DNA would have the highest diagnostic or prognostic utility, perhaps in some pathological or stress-induced states that would produce more DNA for isolation and analysis. Here, we provide a first step towards elucidating the mechanism of exosomal DNA packaging.

Exosomes as DNA Damage Response Mediators

For many years, studies have shown that radiation treatment can induce bystander DNA damage responses (DDR) in treatment naïve cells[309]. While some molecular mediators, such as ROS[326], have been implicated in these effects, the molecular mechanisms of bystander responses remain unclear. Here, we demonstrate that exosomes from cancer cells with induced DNA damage can activate DDR in treatment-naïve cells, thus serving to propagate cellular stress signals. It has been shown that several tumor types can influence the microenvironment with pro-tumorigenic secretory signaling in times of cytotoxic stress[327]. We identify exosomes as a vehicle of intercellular communication, to transfer DNA damage stress responses between cancer cells and both malignant and non-malignant cells in the tumor microenvironment. Potentially, exosome-transmitted DDR can also be communicated

to peripheral organs, including myeloid cells of immune system, causing the formation of pre-metastatic niches or inducing pro-tumorigenic pathways. Indeed, it has been shown that genotoxic cancer treatments can activate DDR programs in naïve cells, leading to the generation of a pro-angiogenic, pro-inflammatory environments that are tumor permissive[328]. Thus, exosome-assisted transmission of DDR could cause enhanced treatment resistance, tumor growth, and metastasis. Identifying the mechanisms by which exosomes mediate DDR activation could provide opportunities for interventions to increase treatment efficacy, such as inhibiting exosome release during chemotherapy or radiation.

Interestingly, non-malignant cells and undamaged cancer cells (not exposed to DNA damaging agents) were incapable of DDR initiation in naïve cells, suggesting that exosomes only acquire DDR-inducing cargo in cancer and DDR-specific contexts. One potential mechanism that we have begun to investigate is the presence of replication fork-protection defects in cancer cells that could lead to tumor-specific responses to DNA damage. This work was initiated in collaboration with Venkateswarlu Popuri, when we observed that only exosomes I generated from tumorigenic cell lines, not non-tumorigenic lines, could elicit DDR signaling in naïve cells. Additionally, non-tumorigenic cells may be more resistant to exosome uptake, which could also explain the lack of increased DDR activation in those cells, necessitating experiments with labeled exosomes to visualize whether exosomes preferentially localize within malignant cells. This evidence supports the hypothesis that cancer cells can utilize exosomes as a survival mechanism to enable treatment resistance by evading DNA damage related death or senescence.

Exosomes Carry Activated DNA Damage Response Pathway Proteins

While our group and others generated data that implicate exosomes in bystander DDR activation[320,329], the mechanisms by which exosomes mediate these effects remain unknown. Here, we show for the first time that exosomes from cancer cells with induced DNA damage contain activated (phosphorylated) DDR pathway proteins, specifically CHK1 and CHK2. Up to this point, no other groups had observed the paracrine transfer of activated DDR proteins, which could potentially induce horizontal activation of the DDR cascade upon delivery to recipient cells. While this initial data is promising, additional experimental validation of these results is required, such as an assessment of total protein in comparison to phosphorylated protein alone, as well as densitometry measurements for more accurate quantification. Additionally, the levels of both total and phosphorylated protein need to be assessed in the recipient cells, to demonstrate the functional transfer of these proteins. Further clarification of the role of exosomes, specifically their cargo, in mediating DDR-inducing cancer cell signaling could help clarify their roles in tumor-promoting programs such as treatment resistance, enhanced growth, and survival. Manipulating exosome release or cargo sorting could negatively impact tumor-promoting responses in the microenvironment after genotoxic treatment, thus mitigating their protective effects and enhancing treatment efficacy.

Future Directions

The future indications for this work involve further investigation into molecular mechanisms of exosomal DNA packaging, exosome-transmitted DNA damage response, and clinical applications of exosomal DNA as a biomarker source. My

preliminary results suggest that exosomes could be used to assess the epigenetic status of patient tumors and thus identify additional therapeutic targets and predict treatment responsiveness. The primary short-term goal will be to continue to optimize exosome isolation and DNA extraction from patient samples, including serum and potentially other biofluids such as urine, to maximize DNA yield and quality for downstream analyses, like sequencing or methylation sequencing, for both genomic and epigenomic alterations. Subsequently, the development of a predictive biomarker assay for tumor types that are difficult to detect early, such as PDAC, could allow for patient-derived exosomal DNA sequencing data to inform early diagnostic capabilities.

Further work is also required for elucidating the detailed molecular and biological mechanisms of exosome-mediated DDR activation. We would like to investigate the cancer-specific nature of exosome-mediated DDR induction by utilizing isogenic cell lines transformed with different oncogenes, such as Kras, to better characterize the DDR induction capabilities of exosomes from transformed vs non-transformed cells. By performing a more detailed analysis of DDR pathway-related exosome cargo and identifying how cancer cells can co-opt exosomes for stress response signaling, potential targets for therapeutic intervention could be discovered. Work could include an assessment of the effects of inhibiting exosome release on DDR activation in naïve cells, or investigating whether cells with exosome-induced DDR become more resistant to therapy or death signaling. Much remains to be discovered regarding the roles of exosomes in patient treatment responses.

Exosome biogenesis and function is still a nascent field of study, where relatively little is known regarding their physiological and pathological roles. Based on

recent work demonstrating the transfer of functional molecules, including DNA, to target cells via exosomes, there is considerable reason to believe that exosomes could mediate an even broader range of paracrine effects. Exosomes travel easily throughout the body and are released and taken up by all cell types, enabling their potential involvement in a variety of functions, including DNA damage response activation. Therefore, the future goals of this research will be to explore a potential role for exosomes in improving diagnostic capacities, as well as investigating their involvement in drug induced chemoresistance and tumor progression, all major barriers for current cancer treatment efficacy. This research provides the foundation for the advancement of therapeutic modalities using exosomes, as they can be easily detected, targeted, and manipulated.

Conclusion

The main goal of this body of work was to gain more information about the origin, function, and utility of exosomal DNA. As I hope I have conveyed, exosomal DNA is packaged into exosomes in a cell type-specific manner and released into the extracellular environment, where it can subsequently be transferred to recipient cells or isolated for analysis. Exosome DNA has tremendous potential for therapy, both in cancer and other diseases, as a diagnostic and monitoring tool. As more information about exosomes is discovered, I believe that it is likely they will become important factors in many therapies and detection assays. It is a privilege to contribute my work to such an exciting and translationally relevant field of biology.

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