Title

Chapter Nine Extracellular vesicles in tumor immunotherapy

ISBN

9780323857819

Authors

Li, Jiawei

Stephens, Elisa

Zhang, Yong

Publication Date

2022

DOI

10.1016/b978-0-323-85781-9.00009-9

Peer reviewed

Extracellular Vesicles in Tumor Immunotherapy

Jiawei Li[1,#], Elisa Stephens[1,#], and Yong Zhang[1,2,3,4*]

[1]Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90089
[2]Department of Chemistry, Dornsife College of Letters, Arts and Sciences, University of Southern California, Los Angeles, CA 90089
[3]Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA 90089
[4]Research Center for Liver Diseases, University of Southern California, Los Angeles, CA 90089
[#]These authors contributed equally to this work
*Email: yongz@usc.edu

Abstract

As endogenous nanocarriers and mediators of cell-to-cell communication, extracellular vesicles (EVs) have been emerging as promising therapeutic agents to combat many diseases. Of the different categories of EVs, exosomes have gained particular prowess in therapeutic development due to valuable pharmacological properties. Exosomes are small membranous vesicles secreted from diverse types of cells, and upon their biogenesis, they inherit contents including proteins, DNA fragments and RNAs from parental cells. The subsequent release of exosomes into the extracellular space plays an important role in intercellular communication by initiating signaling through inherited surface ligands and transferring cargo contents upon uptake. Tumor-derived exosomes have been shown to be critical for tumor metastasis, angiogenesis, and microenvironmental modulation through these processes. Moreover, given their abilities to modulate the immune system, exosomes have also become important candidates for immunotherapy. In this chapter, the biogenesis, physiological functions, and roles in immune modulation for exosomes are first introduced. Exosome-based cancer applications are then highlighted such as cancer vaccines, exosome as delivery vehicles of therapeutic cargos, and current clinical and translational research. Lastly, the future development of exosomes in immunotherapy is discussed.

Keywords: extracellular vesicles, exosomes, exosome biogenesis, intercellular communication, immune modulation, cancer, immunotherapy, vaccines, drug delivery.

Introduction

Extracellular vesicles (EVs) have been emerging as promising tools for the study and treatment of various diseases due to their extensive involvement in numerous physiological and pathological processes, from tissue and immune regulation to pathogenic injury and cancer. As a heterogenous group of cell-derived membranous vesicles, EVs can be secreted by a variety of cells and are found in most bodily fluids.[1,2] Though originally described as a means of eliminating cellular waste,[3] EVs are produced for purposes beyond simple component recycling. Importantly, EVs are shown to exchange contents between cells and act as signaling vehicles to mediate intercellular communication in health and disease.[4,5,6] Studies of EVs have been mainly aimed at their classification, isolation, functional characterization, and, most recently, biomedical application.[7] Due to the morphological and functional heterogeneity of EVs, the physiological relevance of purported EV-mediated interactions remains unclear and is further exacerbated by difficulties in achieving pure preparations and proper characterizations.[8] Nevertheless, as endogenous nanocarriers and mediators of cell-to-cell communication, EVs provide promising utility as cell-free diagnostic and therapeutic agents.

Though classification techniques are evolving, EVs can be broadly categorized into two main groups: ectosomes and exosomes, based on current knowledge of their biogenesis. Ectosomes are vesicles that bud directly from the plasma membrane of the cell and include microvesicles, microparticles, and large particles that can range from 50 nm to 1 μm in size. Exosomes are vesicles of endosomal origin and are secreted from the cell upon exocytosis of multivesicular bodies (MVBs). Because they originate from the endosomal pathway, exosomes are generally limited in size from 50 nm to 200 nm. Nonetheless, exosomes possess important biological and pharmacological properties that make them ideal nanocarriers for the delivery of therapeutic cargos.[9] In comparison to viral and synthetic nanocarriers, endogenously derived exosomes may exhibit reduced immunogenicity. Exosomes commonly carry enriched membrane proteins that promote membrane fusion with target cells, facilitating cellular delivery of exosomal cargos.

Of particular importance, tumor-derived exosomes (TEXs) can present tumor-associated antigens either directly or indirectly through antigen presenting cells (APCs) to immune cells, eliciting antitumor immunity.[10-12] However, the

tumor cells also sneakily pack ligands and RNAs that may induce metastasis, angiogenesis, and immunosuppression.[13-16] These malignant biomarkers on TEXs may provide non-invasive tools for both cancer diagnosis and prognosis.[17] To contour the immunosuppressive dilemma of TEXs, the APCs could produce exosomes with less tumorigenic tendency and improved safety profiles.[18-20] In addition to these endogenous exosomes, modifications to the cargos and surface ligands of TEXs and dendritic cell-derived exosomes (DEXs) were attempted to increase the antitumor efficacy. Furthermore, development of bi- and multi-functional exosomes may build a platform for targeted cancer immunotherapy.

This chapter is focused on exosomes and the attributes that make them exciting contenders in the race to discover novel therapeutic agents for tumor immunotherapy. Specifically, current knowledge on exosome morphology and biological function are summarized with an emphasis on the critical roles exosomes play in mediating cellular communication within the immune system. The biomedical applicability of native and engineered exosomes in cancer immunotherapy and their challenges in clinical development are discussed, followed by a discourse on the advantages and disadvantages of using exosomes as immunotherapeutics and future directions in the field.

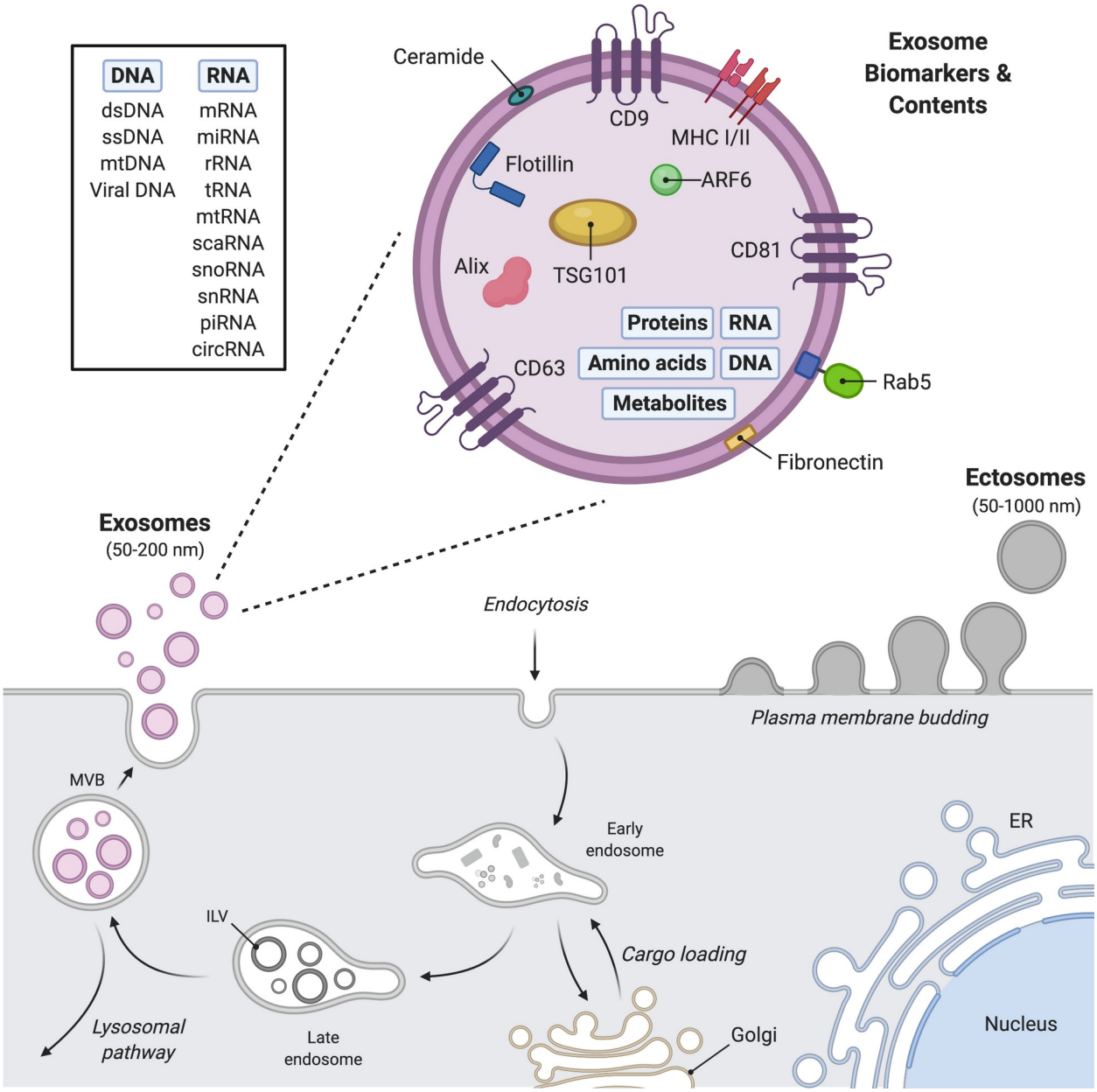

Figure 1. Biogenesis of exosomes. Along the endosomal pathway, future exosomes (known as ILVs) are loaded with cytoplasmic constituents. Late endosomes containing fully formed ILVs give rise to MVBs, which can either be transported back to the plasma membrane or degraded via the lysosomal pathway. Upon successful docking to the plasma membrane, MVBs release exosomes into the extracellular space. Exosomes commonly contain several proteins known to be involved in their biogenesis such as Rab GTPases and ESCRT proteins, as well as surface proteins like tetraspanins, integrins, and immunomodulatory proteins. Other common exosome biomarkers include flotillin, TSG101, ceramide, and Alix. Exosomal cargos vary widely depending

on the originating cell and include proteins, RNA, DNA, amino acids, and metabolites.

Biogenesis and Biological Functions of Exosomes

Exosome Biogenesis and Morphology

As a dynamic, multi-faceted cellular process, exosome biogenesis involves two rounds of invaginations of the plasma membrane (Figure 1). The first invagination event occurs at the plasma membrane itself via endocytosis, a process that forms a distinct cup-shaped structure that is lined with cell-surface proteins and contains soluble components of the extracellular milieu. This structure eventually buds off inside the cell to form a membrane-bound vesicle known as an early-sorting endosome or simply an early endosome. The content composition of early endosomes is largely dependent on the type of cell and the environment of the cell, but it can also be influenced by the trans-Golgi network and endoplasmic reticulum (ER), which can play roles in vesicle formation and content loading.[1,6,21] Over time, the early endosome matures into a distinct structure known as a late-sorting endosome (late endosome) that is capable of producing new membrane-bound structures known as intraluminal vesicles (ILVs). ILVs are formed upon inward invagination of the late-endosomal membrane (a second invagination of the plasma membrane) and contain directly sorted as well as acquired cytoplasmic and membrane-bound contents. Upon ILV formation, late endosomes are referred to as multivesicular bodies (MVBs**)**. At this point in the endosomal pathway, MVBs have one of two fates: degradation via lysosomes or transport back to the plasma membrane. Fusion of MVBs with the plasma membrane facilitates the exocytosis of ILVs into the extracellular space as exosomes.

Given that exosomal content is dependent on parent-cell type and cellular environment, exosomes are an inherently heterogeneous population of EVs. For example, cancerous cells tend to release exosomes decorated with tumor-specific antigens and packaged with distinct miRNAs compared to normal cells.[22,23] Furthermore, the activation state of the parent cell, which is directly influenced by its environment, can result in the secretion of unique sub-populations of exosomes.[24] However, despite this morphological heterogeneity, exosomes possess a defined protein and lipid composition that helps to distinguish them from other EVs (Figure 1). Unsurprisingly, exosomes are typically enriched with proteins and lipids that are directly involved in their biogenesis and release, such as the Ras-related protein

GTPase Rab, the small GTPase ADP ribosylation factor 6 (Arf6), TSG101 (tumor susceptibility gene 101), ESCRT (endosomal sorting complexes required for transport) proteins, Alix (apoptosis-linked gene 2-interacting protein X), flotillin, and ceramide. In addition, the membranes of exosomes tend to be highly enriched with tetraspanins like CD9, CD63, CD81, and CD82, which are transmembrane proteins known to regulate protein sorting into EVs.[25] Other common exosomal marker molecules include heat shock proteins (Hsp70 and Hsp90), which are expressed by cells under conditions of stress, integrins, and immunomodulatory proteins like MHC class I/II (major histocompatibility complexes class I and II).

While these particular biomarkers are helpful in distinguishing exosomes from other EVs, it is important to keep in mind that exosomes can vary widely from one another depending on their size, cargo, and relative expression of cell surface receptors. Size inequalities likely arise due to uneven invaginations of the late-endosomal membrane, resulting in differential total fluid and solid exosomal contents.[7,26] Similarly, different cell types exhibit varying patterns of protein, metabolite, and nucleic acid expression, which can influence exosomal loading of these species. Importantly, these expression patterns can be further altered depending on the specific microenvironment of the cell. Taken together, the complex architecture of exosomes implies a wide range of possible functions once released into the extracellular space.

Functions of Exosomes

Shortly after their classification in the 1980s, exosomes were first reported to serve as a biological means of shedding proteins and other cellular components like RNA that are no longer needed by the parent cell.[3,27,28] Specifically, exosome formation was proposed as a major route of removal of plasma membrane proteins during reticulocyte maturation and plasma membrane remodeling. This discovery branded exosomes as the “garbage cans” of cells, and research in the field was at a standstill for nearly a decade. Nevertheless, as more and more cell types were shown to secrete these EVs, scientific interest in further elucidating exosome function reignited. Importantly, Raposo and colleagues found that exosomes play an active role in intercellular communication within the immune system.[29] It was shown that cultured B cell lines secreted exosomes containing peptide-bound MHC class II could induce antigen-specific, MHC class II-restricted T cell responses. Similarly, Valadi and coworkers showed that exosomes derived

from mouse and human mast cells contain mRNAs capable of being delivered to other mast cells and translated into functional proteins.[30] Collectively, these findings changed the narrative of exosome function and established exosomes as critical mediators of intercellular communication and as signal transducers.

Exosomes contain unique biological features that make them ideal facilitators of cell-to-cell communication. Notably, the phospholipid bilayer of exosomes is abundantly decorated with the tetraspanin CD9, a transmembrane protein that promotes direct membrane fusion with recipient cells, thus, facilitating cellular delivery of exosomal cargos.[25] In addition, the transmembrane protein CD47 prevents clearance of exosomes by monocytes and macrophages, allowing them to circulate longer and distribute more.[31] Other proteins, such as integrins and immunoglobulins, have also been shown to play important roles in cell-to-cell adhesion, antigen presentation, and cell signaling upon direct contact with the target cells.[32] Though shared features like these help promote initial contact and fusion with cells, unique exosomal features such as cargo type and surface decorations, both of which are governed by the parent cell, are the major determinants of the phenotypic and molecular alterations induced by these interactions. Hence, specific exosome function is largely dependent on: 1) the extent of exosome uptake by the target cell and 2) the materials that are transferred in the process. Importantly, different recipient cells may rely on different mechanisms of exosome uptake. For example, exosome uptake by human pancreatic cancer cells is facilitated by CD47-dependent macropinocytosis, while uptake by cells derived from rat adrenal medullary tumors is reliant on clathrin-dependent endocytosis.[31,33,34] The extent of exosome uptake by target cells can be further influenced by varied expression of cell surface receptors, resulting in varied levels of effects. Similarly, the identity of the cargo that is delivered to recipient cells upon exosome uptake impacts the effect on target cells and, thus, exosome function. For instance, secretion of RNA cargos via exosomes can result in altered gene regulation and expression in the recipient cells, while transfer of protein cargos can result in altered cell signaling that can lead to a wide range of effects including immunomodulation, tumorigenesis, and apoptosis.[26,35,36] Collectively, these studies demonstrate that exosomes are capable of selectively inducing signals in target cells to regulate cellular processes such as immune responses, cell proliferation, and differentiation.

Role of Exosomes in Immune Regulation

Since exosomes were reported to activate T cells in the late 1990s, their role in immune responses has been widely documented. Shortly after the discovery that B cell derived-exosomes carry peptide-bound MHC class II and induce T cell responses,[29] Zitvogel and colleagues showed that dendritic cells (DCs) secrete antigen-presenting exosomes that are capable of stimulating antitumor T cell responses *in vivo*, thus, improving tumor eradication in mouse models.[37] Similar results reported by a separate group a few years later revealed that endogenous APCs must efficiently uptake these MHC class II-bearing exosomes in order to initiate T cell activation.[38] Hence, these early findings helped establish the role of exosomes in eliciting adaptive and innate immune responses.

The different mechanisms that exosomes use to elicit immune responses are largely cargo-dependent and, thus, are heavily influenced by the identity of the originating cell (Figure 2). Exosomes from APCs like B cells, DCs, and macrophages tend to carry peptide-bound MHC class I and/or MHC class II along with costimulatory signals from the parent cells. These surface decorations allow APC derived-exosomes to directly present peptide antigens to immune cells, thus, inducing their activation. In fact, exosomes derived from human DCs have been shown to promote both T helper and cytotoxic T cell responses *in vitro* and *in vivo*.[39,40] Importantly, the surfaces of these DC-derived exosomes were found to be enriched in known immune regulators like CD40, CD209, CD80, CD86, and CD54. On the other hand, exosomes derived from mesenchymal stem cells (MSCs), tend to exert immunosuppressive effects via a variety of immunomodulatory effectors like transcriptional factors, non-coding RNA species, and cytokines. As a result, MSC-derived exosomes have a propensity to inhibit B cell activity and DC maturation.[41,42] For example, MSC-derived exosomes were shown to not only increase the release of the immunosuppressive cytokine interleukin 10 (IL-10), but also increase the ratio between T regulatory cells and effector T cells.[43] Likewise, exosomes derived from tumor cells also tend to exert immunosuppressive effects, thus, promoting tumor growth, metastasis, and, in some cases, the development of drug resistance. Tumor-derived exosomes can achieve this by presenting immunomodulatory molecules like PD-L1 (programmed cell death ligand 1) and FasL (Fas cell surface death receptor ligand) on their surfaces. For instance, glioblastoma-derived stem cells produce exosomes with upregulated PD-L1, thus, inducing a STAT3-mediated switch of macrophage phenotype towards type 2 and creating an immunosuppressive microenvironment.[44] Similarly, exosomes derived from a

human prostate cancer cell line were shown to express FasL, thus, stimulating Fas-dependent T cell apoptosis.[45] Tumor-derived exosomes can also achieve tumor immune evasion by influencing gene expression in target cells. Exosomal miRNAs derived from pancreatic cancer exosomes could transfer to dendritic cells and inhibit expression of RFXAP, an important transcription factor for MHC class II.[46]

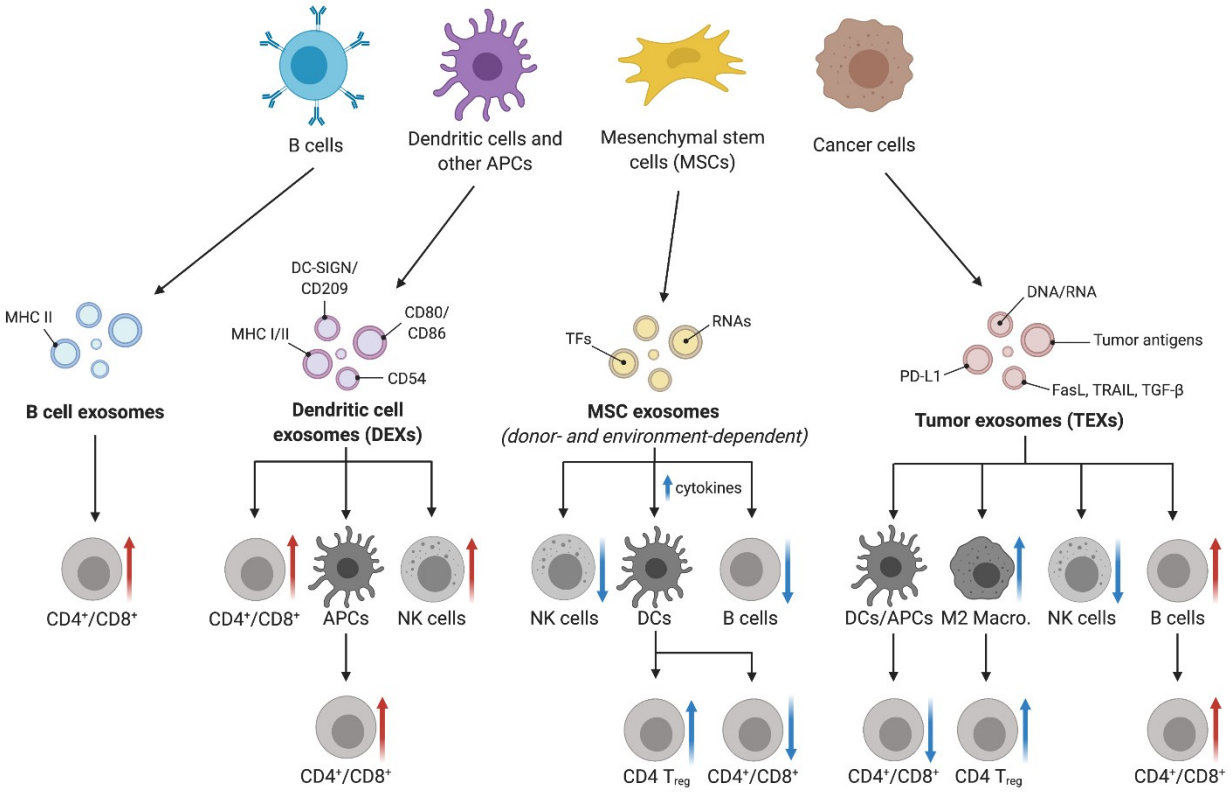

Figure 2. Immune responses elicited by exosomes. The distinct cellular source of exosomes can directly influence their abilities to regulate the innate and adaptive immune system. Cellular sources of immunomodulatory exosomes include B cells, DCs, MSCs, and cancer cells.[7] Exosomes derived from these sources can both directly and indirectly regulate the activity and proliferation of effector T cells ($CD4^+$, $CD8^+$, and CD4 T_{reg}), natural killer (NK) cells, and other immune players like B cells, DCs, and other APCs. The direction of arrows indicates relative expression/activity (arrow up = higher expression/activity, arrow down = lower expression/activity) and the color of the arrow indicates net immune effects (red = immune activation, blue = immune suppression).

Though studies on tumor-derived exosomes have primarily reported immunosuppressive outcomes, there have also been some reports of immune activation and tumor suppression. Importantly, tumor-derived exosomes present tumor antigens that can activate antitumor immune responses by B cells and effector T cells. In one such study, tumor-derived exosomes were used as a source of tumor-rejection antigens to prime DCs, thus, inducing cytotoxic T cell-dependent antitumor effects in mice.[47] These results were echoed in a similar DC-priming study that demonstrated the ability of exosomes derived from human malignant effusions to induce antigen-specific cytotoxic T cell responses.[48]

Given the abilities of exosomes to both induce and suppress innate and adaptive immune responses, their therapeutic potential is widely explored and discussed extensively in the following sections.

Exosome Immunotherapy Applications

DEXs and TEXs: Cancer Vaccines

One of the most studied areas for exosomes in cancer immunotherapy are cancer vaccines, which can be categorized into two types: DEXs and TEXs. Despite the fact that TEXs-based vaccines remain at the preclinical stage, clinical trials using ascites-derived exosomes as cancer vaccines may forecast clinical performance of TEXs. Clinical trials for DEXs were completed, with one in phase II and others in phase I[19]. However, their outcomes suggested that further improvements are required.

The pharmacological functions of DEXs in immune modulation are closely associated with DCs. DEXs could activate T cells through both MHC complexes and the costimulatory molecules inherited from the parental DCs.[49-50] Following stimulation, T cells could expand and initiate antigen-specific responses.[19, 51-52] T-cell stimulation via DEXs might include several paths. Despite the fact that DEXs could not directly prime naive T cells effectively[53] and are unable to activate T cells in the absence of APCs,[54-55]{Thery, 2002 #76} DEXs are effective in stimulating preactivated T cells, T-cell hybridomas, clones, and cell lines, which typically require less extent of T-cell receptor (TCR) crosslinking than naive T cells.[19, 56-57] DEXs could bind to bystander DCs for indirect stimulation with assistance from exosomal surface proteins including integrins and ICAM-1.[57-58] In addition, DEXs could pass on the antigens to bystander DCs by means of phagocytosis or

macropinocytosis,[19] which facilitate displaying of MHC-tumor peptide complexes on the plasma membrane of DEXs for efficient presentation to T cells.[51, 59-60] DEXs-treated tumor cells are more susceptible to immune attack, suggesting an alternative route of indirect T-cell activation by DEXs.[61]

Despite the fact that multiple studies reported the malignant effects of TEXs on cancer progression, tumor microenvironment regulation, immune modulation, and cancer therapy resistance,[13, 15-16, 62] TEXs' potential as cancer vaccines is not negligible. TEXs are known to serve as important communicators among cancer cells, immune system, and local and distant organs.[63-64] Upon secretion from tumor cells, TEXs could penetrate into local tissues, promoting angiogenesis by translating cell cycle-related mRNAs.[65] TEXs also contain diverse pro-angiogenic miRNAs and cytokines, activating angiogenesis-related cellular pathways.[66-67] The hypoxic microenvironments of tumors could promote secretion of pro-angiogenic factors-containing exosomes to enhance tumor cell survival,[68] which could in turn aggravate the tumorigenic microenvironment. Distantly, TEXs could travel through blood, forming pre-metastatic niches supporting tumor microenvironment at other organs.[69] While the delivered miRNAs could target the angiogenic pathway to facilitate cellular migratory and invasion ability,[70] the proteases carried by TEXs could degrade epithelial adhesion molecules, promoting tumor progression.[71] In addition, TEXs were shown to induce chemotherapy drug resistance by exporting drugs through exosomal secretion, resulting from drug-induced phenotypic changes.[72]

Given the different functions and roles of TEXs in tumor immunology, it is difficult to draw a clear line between the immunosuppression and immunoactivation of TEXs. Secreted by tumor cells, TEXs are naturally abundant in tumor antigens. Through internalization into DCs, the tumor antigens from TEXs are sorted and bound with MHC class I and II molecules. Similar to the indirect antigen presentation by DEXs, antigens from TEXs could also be presented to T cells via DCs.[11] NK cells proliferation and cytotoxic responses were shown to be inhibited by TEXs through IL-2 blocking.[73] Inhibition of IL-2 could also enhance the proliferation of T_{reg} cells, suppressing the expansion of other $CD4^+$ T cells, which could affect other T cell subsets but not $CD8^+$ cells.[74] TEXs can also induce the loss of CD27/28 on $CD8^+$ T cells, which subsequently lead to suppressor phenotype with immune dysfunction.[75] Myeloid-derived suppressor cells (MDSCs) are highly associated with the tumor disease state. TEXs were found to drive the myeloid cells' differentiation into MDSCs by inhibiting DCs formation.[76] The

enlarged population of MDSCs could further foster a tumorigenic microenvironment and increase anti-apoptotic protein expression in B cells.

Several unmodified TEXs were examined in mouse models and exhibited T-cell dependent antitumor responses.[11] Bu and colleagues showed that vaccination with exosomes derived from lymphocytic leukemia cells could induce antigen-specific T-cell response and inhibit tumor growth.[77] In another study, exosomes derived from brain tumor cell lines were vaccinated in mice to evaluate their functions in immune modulation. Within TEXs-treated group, 80% mice revealed no tumor development, whereas all mice in the control group died after 55 days of tumor implantation.[78] In the pre-established tumor models, TEXs provide no benefits to the overall survival, albeit a low amount of antibodies against the exosomes were detected.

In order to improve efficacy, a range of manipulations were attempted on TEXs. Studies indicated that heat shock proteins (HSPs) are highly associated with the antitumor immunity due to their roles in the interactions with APCs as well as the immune recognition.[79-80] Tumor cells undergoing a heat shock process could drive higher levels of HSP expression on exosome surface. Cho *et al.* compared the exosomes from the heat-treated tumor cells and the non-treated ones. The superior tumor immune responses in both allogeneic and autologous *in vivo* mouse tumor models administered with heat-treated TEXs were attributed to the increased HSP70 expression.[81] More than heat treatment, HSP70 expression could be increased through genetic engineering. By designing a HSP70 fusion with a membrane-binding region, the HSP70 expressed on the membrane could be more abundant than one in cytoplasm. The genetically modified exosomes enriched with HSP70[82] were shown to induce stronger T-cell and NK-cell responses than the heat-treated TEXs, implicating that surface HSP70 may act as both an alerting signal for the immune system and an antigenic peptide. Chemotherapy drugs could also trigger increased HSP expression on TEXs, leading to enhanced NK-cell response.[83]

Genetic manipulation of source tumor cells could increase the expression of antigenic moiety on TEXs surface, hence improving anti-tumor efficacy. By introducing MUC1,[84] a tumor antigen associated with cancer progression, the resulting TEXs foster the maturation of DCs. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can activate the apoptotic pathway in cancer cells. Exosomes derived from TRAIL-introduced leukemia cells were

shown to reduce lymphoma tumor growth *in vivo* and induce apoptosis for several types of cancer cell lines *in vitro.*[85]

Engineering the cargos of TEXs, especially immunomodulatory miRNAs, could also alter the anti-tumor activity. Increased expression of miR-9 in glioblastoma multiforme leads to resistance to chemotherapy, while delivery of anti-miR-9 could hinder the expression of a multidrug transporter, resuming cellular sensitivity towards temozolomide.[86] In another example, genetic engineering of breast cancer cells with tumor suppressor miR-134 produced TEXs enriched with miR-134. Cancer cells treated with the modified TEXs showed reduced metastatic potential and resistance to anti-HSP90 drugs.[87]

In addition to using TEXs as vaccines, TEXs can serve as an antigen source for DEXs. The DEXs produced by DCs and loaded with TEXs could prolong the survival of tumor-bearing mice in comparison with lysate-loaded DEXs. The augmented antigen-specific $CD4^+$ T cell response was characterized by trogocytosis and proliferation. TEXs could be efficiently taken by DCs, especially through the MHC class II-loading compartment, leading to a longer antigen presentation time and extended recovery by T cells. No DC phenotypic change resulted from the TEXs was observed.[88]

Directly applying unmodified TEXs for cancer therapy may be risky given their tumorigenic nature. By loading with immunostimulatory molecules or deactivating the malignant components, the anticancer activity and safety profile of TEXs could be enhanced, enabling future clinical applications.

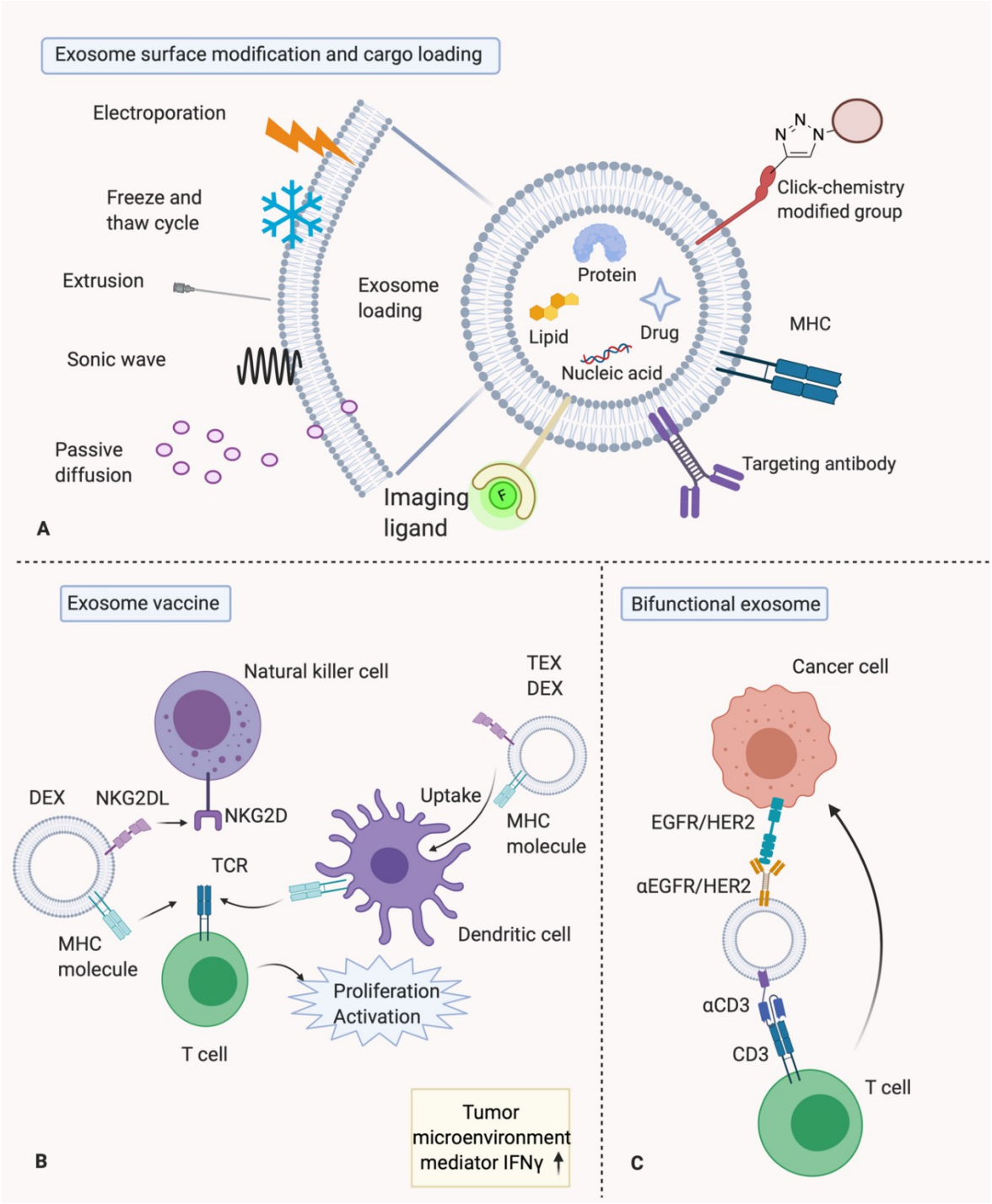

Figure 3. Native and engineered exosomes for tumor immunotherapy. 3A: exosomes can be loaded with proteins, lipids, small-molecule drugs, and nucleic acids by means of electroporation, freeze and thaw cycle, extrusion, sonication, and passive diffusion.[89] 3B: TEXs and DEXs can be internalized by DCs for antigen presentation to immune cells. DEXs may also directly interact with T cells and NK cells.[11, 18] 3C: bifunctional exosomes, also termed as SMART-Exos, could dually target cancer cells and

T cells, promoting the formation of immunological synapses for tumor elimination.

Exosomes Engineered with Proteins/Peptides

Exosomes could be armed with immunomodulatory ligands through genetic engineering. IL-18[90] can act as a vaccine adjuvant to promote T cell proliferation ad generation of immunostimulatory cytokines by activating lymphocytes. Through transducing a human colon adenocarcinoma cell line with high levels of carcinoembryonic antigen with IL-18 gene, exosomes with expressed IL-18 could be generated. In addition to increasing Th1 and TNF-α production, IL-18-expressing exosomes could chemoattract DCs and T cells and promote the maturation of DCs. In another study, ovalbumin antigen-positive tumor cells were genetically engineered to express IL-2.[91] The resulting exosomes could induce anti-tumor effects and act as an immunological promoter for immune cells.

Functional proteins and peptides could also be docked on the membranes after co-incubation of exosome-producing cells. The generation and presentation of MHC-tumor antigen peptides complexes by DCs are essential for priming cytotoxic T lymphocytes.[49] MHC-peptides complex-containing exosomes were harvested following the incubation of DCs with acid-eluted tumor peptides.[49, 92] The peptide-loaded DEXs exhibited higher *in vivo* anti-tumor efficacy, achieving suppression or even eradication of tumors, than the tumor-peptide loaded whole DCs. The superior activity of DEXs might be attributed to the stable levels of costimulatory molecules presented on DEXs, which might be downregulated on DCs due to phenotypic changes in tumor microenvironments, signifying exosomes' advantage as a cell-free therapeutic alternative. Post exosomes production, peptides can also be loaded by simple co-incubation. KLA,[93] a 14-amino acids peptide, was shown to exert antitumor activity by inducing cell apoptosis. The exosomes derived from fibroblast cells were first loaded with methotrexate, followed by conjugation with the KLA through incubation with the assistance from ApoA-I mimetic peptide. The resulting exosomes could significantly extend the survival of tumor-bearing mice relative to the methotrexate-loaded exosomes. Alternatively, attaching superantigen staphylococcal enterotoxin A (SEA) with a hydrophobic tail facilitates direct insertion into exosome membrane.[94] SEA-attached TEXs could induce tumor-specific T-cell responses.

A novel class of bioengineered exosomes, termed as synthetic multivalent antibodies retargeted exosomes (SMART-Exos)[95-98](Figure 3C), were generated for recruiting and activating cancer-specific T cells. SMART-Exos are characterized by surface-displayed antibody fusions specific for tumor-associated antigen and T-cell CD3. Monoclonal antibodies were genetically fused and anchored on the exosome membrane through the transmembrane domain of human platelet-derived growth factor receptor (PDGFR). The SMART-Exos could foster the formation of immune synapses between tumor cells and T cells, redirecting cytotoxic T cells to act on cancer cells. SMART-Exos were demonstrated with excellent *in vivo* effectiveness for established tumors, providing a new and versatile approach for cancer immunotherapy.

Exosomes Engineered with RNAs

Previous studies indicated that transfer of exosomal RNAs could modulate tumor microenvironments, promoting tumor growth, metastasis, and angiogenesis.[99-100] On the other hand, exosomes also assist the delivery of RNA cargos to cancer cells for gene expression regulation and for targeting the tumorigenic pathways.[101-102] Brain endothelial cell-derived exosomes loaded with siRNAs targeting vascular endothelial growth factor (VEGF) could cross blood-brain barrier to suppress the levels of VEGF mRNA and protein.[103] In a xenograft zebrafish brain tumor model, decreased tumor fluorescence intensity was observed in the exosome treatment group. Breast epithelial cells-derived exosomes loaded with CDK4 siRNAs could efficiently decrease the levels of CDK4 mRNA and protein *in vitro* and *in vivo*, slowing cancer progression.[104] Tumor RNAs carried by cationic liposomes (lipoplex) could elicit immune responses through uptake by macrophages and DCs, triggering release of IFNα. By displaying tumor antigens originated from the tumor RNAs, DCs could induce strong antigen-specific T cell responses and IFNα-dependent rejection of progressive tumors.[105] It need to be noted that liposomes might induce unwanted immune responses and anaphylactic reactions, and the delivery efficiency could be lower than exosomes since liposomes are more susceptible to macrophage clearance.[106]

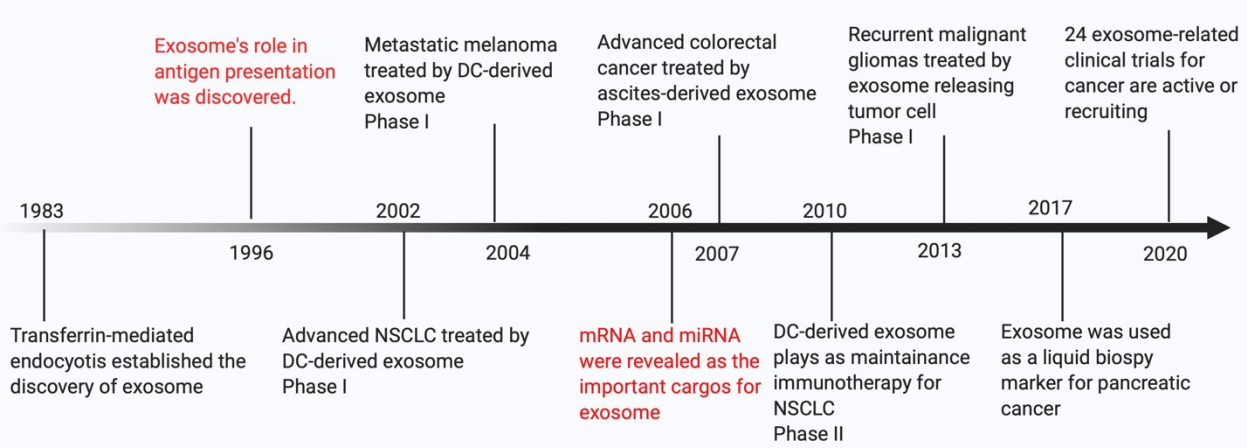

Figure 4: Important discoveries and development related to exosomes.[29, 107-113]

Clinical Development

Clinical Trials

The safety and efficacy for utilizing exosomes in tumor immunotherapy were also examined in several clinical studies. An exosome-based cancer vaccine was examined in a phase I trial for the treatment for advanced non–small cell lung cancers (NSCLC) by DEXs.[109] Of thirteen patients enrolled, nine completed the study. The DEXs were generated by leukapheresis, followed by loading of the tumor antigenic peptide of human melanoma antigen (MAGE) series. The vaccine was administered in four doses and no serious intolerance was observed. Three of the nine patients exhibited MAGE-specific systemic immune responses by delayed type hypersensitivity (DTH) testing. The increased T_{reg} cells populations might suppress cytotoxic T cell responses.

Immediately following this clinical trial, another phase I trial was performed for 15 MAGE3+ advanced malignant melanoma patients.[110] MHC class I or II MAGE molecules were loaded on DEXs by co-culturing with DCs. One objective response according to the RECIST criteria, 2 stable diseases, 1 mixed and 1 minor response were observed. No MAGE3+ specific T cell responses could be detected from patients' blood samples, which might be improved by co-administration of adjuvants or loading with protein antigens instead of peptides. The elevated NK cell responses were observed from 8/13 patients, implying DEXs' efficacy in stimulating NK activity in *vivo.*

Another phase I clinical trial enrolled 40 patients with advanced colorectal carcinoma.[112] Different from the first two, the exosomes were derived from ascites and granulocyte–macrophage colony-stimulating factor (GM-CSF) was co-administered as vaccine adjuvants. The ascites-derived exosomes (Aex) are featured with abundant tumor-associated carcinoembryonic antigen (CEA). CEA-specific T-cell responses were induced with immunostimulatory assistance from GM-CSF. However, besides one stable disease case, no other significant therapeutic response was observed. The ascites provided exosomes of mix sources, including ones from tumor cells. The immune escape property of TEXs might mitigate cytotoxic T lymphocytes responses, which may decrease overall efficacy. No severe adverse event was observed.

To improve the poor outcomes of phase I clinical trials for NSCLC, DCs matured by cytokines IFNγ were used as the source cells to produce DEXs equipped with stronger antigen-presenting abilities and immune response activation potential. A phase II clinical trial recruited 22 patients with advanced unresectable NSCLC.[113] Before administering the DEXs as a less toxic maintaining immunotherapy, platinum-based induction chemotherapy was used as the first-line treatment. The DEXs were derived from healthy donors' DCs instead of autologous sources from patients. Following three weeks of metronomic oral cyclophosphamide (CTX), which suppress T_{reg} cells functions, four exosome vaccinations were administered with weekly intervals. After evaluation of the disease evolution, six biweekly vaccinations were administered, then three weeks interval vaccination, until no exosomes left. Though the vaccinations were safely tolerated by patients, no antigen-specific T-cell responses were detected. The NKp30 ligand BAG6 and MHC class II molecule contained in the vaccines facilitated the enhanced NK cell functions. The primary endpoint for the clinical trial was the reaching of stable disease for more than 4 months. Only 7 out 11 patients for such cases were observed. It was suggested that the peptide loaded on the exosomes might be antigenically irrelevant to the tumors and IFNγ used for maturing DCs could also upregulate exosomal PD-1 expression. Co-administration of immune checkpoint inhibitors might modulate immunosuppressive tumor microenvironments and offset the upregulated PD-1.

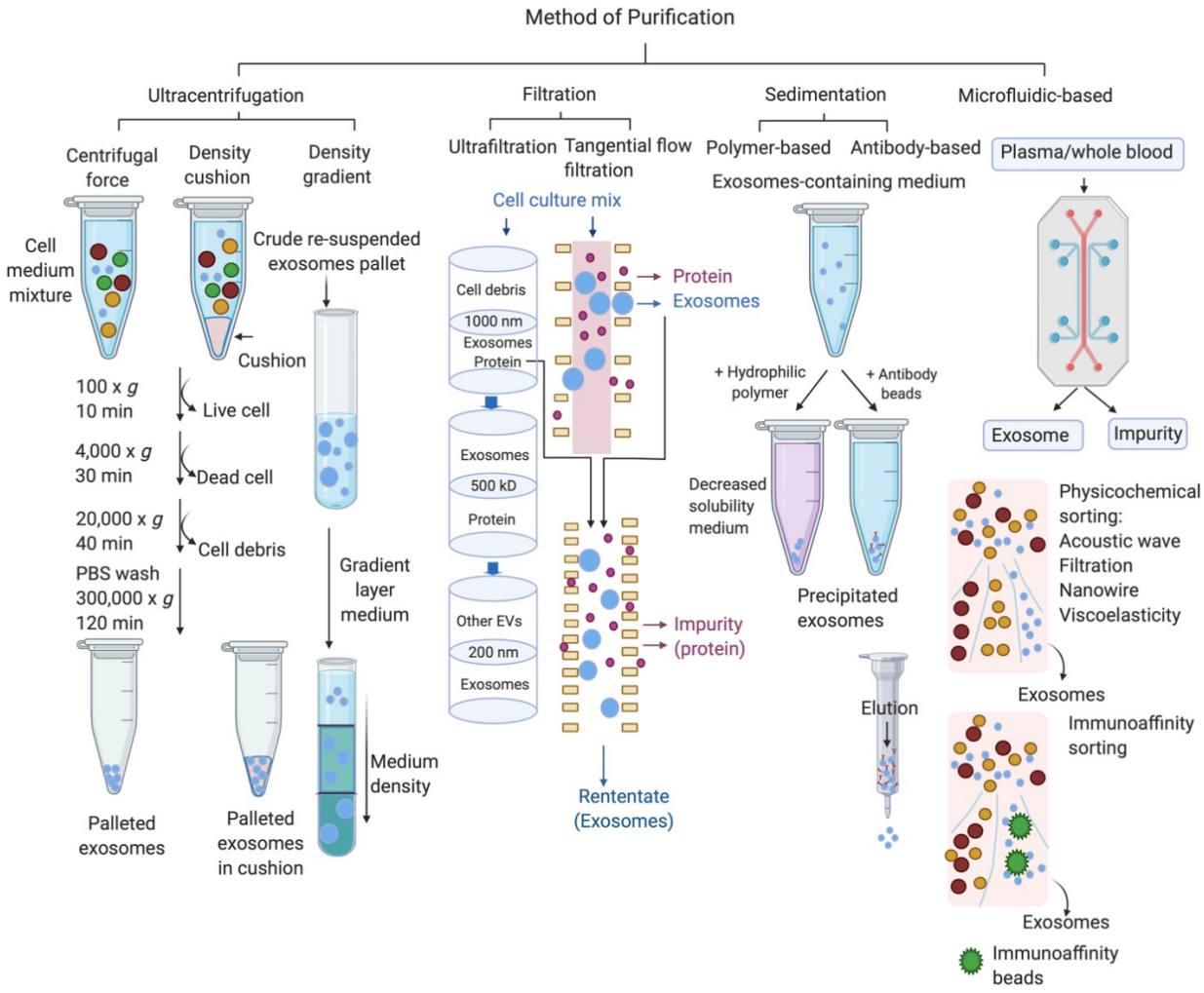

Figure 5: Purification methods for exosomes. Ultracentrifugation: centrifugal force only, density cushion, and density gradient. Filtration: ultrafiltration and tangential flow filtration. Sedimentation: polymer-based and antibody-based methods. Microfluidic-based approach.

Production and Purification of Exosomes

The scales for laboratory production of exosomes are rather small for clinical uses. Translation to production scales compatible with clinical needs might shift exosomes' quality. Large-scale production of exosomes in clinical grade is critical for translating the exosomes-based applications into clinical uses.[114]

Following exosome production, different purification methods are also correlated with the quality and activity of exosomes (Figure 5).

Ultracentrifugation, the most commonly used method can remove cells and cell debris by differential centrifugation. Though ultracentrifugation was used in past clinical trials, the production efficiency is uneconomical in terms of yield, time and cost. Adding density cushions to the medium reduces purification steps with enhanced purity and the cushion protects exosomes' structure integrity by preventing pellet formation at the bottom of the tube and the accompanying high hydrostatic pressure.[115] The density gradient method introduces solutions of a ladder of buoyant density, and the density is created by sugar[116] or iodixanol,[117] which is mostly compatible with the downstream experiments. The density gradient-based method allows the separation between exosomes and other protein contaminants or EVs, improving exosome quality and reducing safety risk.

Ultrafiltration differentiates the components in the medium by the pore size of the membrane with the components of the molecular weight below the weight cutoff going through the membrane and the heavier components trapped above the membrane. The ultrafiltration also requires the vacuum pressure of approximately 517 kPa, which is less damaging for exosomes than the conventional centrifugation method and beneficial for retaining exosome structural integrity.[118] Tangential-flow filtration (TFF) mediates the flow of fluids across the membrane, preventing the dead-end caused by aggregation of biomolecules.[119] TFF allows scalable production and buffer exchange during purification, thus providing an attractive choice for consistent quality exosomes for clinical applications.

PEG solution precipitates exosomes by decreasing the solubility of exosomes in solution.[114] The sedimentation needs lower centrifugal force. But it also introduces PEG and other non-specific precipitated impurities. An improved version of sedimentation utilizes the antibody-coated magnetic beads to isolate exosomes, producing high-purity exosomes with increased yields.[120] Nevertheless, the cost and availability of antibodies and limitation on the exosome surface markers restrict its broader utility.

Microfluidics provides rapid and accurate isolation of exosomes directly from fluids. The separation methodologies are based on filtration, acoustic waves, nanowire trapping, viscoelasticity and immunoaffinity. Exosomes can be categorized into different subsets based on their physical, chemical and biological properties. The microfluidics equipment can be integrated on chips and the nanoscales exosomes production allows liquid biopsy for disease diagnosis instead of large quantity production.

The past clinical trials have demonstrated the utility of ultrafiltration, ultracentrifugation[113] and PEG[121] precipitation in producing clinical-grade exosomes, though their qualities vary from different methods. Efficient and scalable manufacturing approaches that produce consistent quality exosomes will promote the translation from laboratory-based discoveries into broader clinical applications.

Future Opportunities

Exosomes as Diagnostic Tools

Given their wide range of function and tolerability, exosomes can be applied in areas beyond tumor immunotherapies. In particular, exosomes exhibit promising utility as diagnostic tools for different conditions, especially for cancer. As a result of their biogenesis, exosomes have the innate ability to capture the state of a microenvironment, which can be precisely characterized based on the cargos. For example, the DNA cargos found in serum exosomes can aid in detecting cancer-associated mutations in the genome, such as those in *KRAS* and *TP53* found in pancreatic cancer.[122-125] Exosomal miRNAs also prove potentially beneficial in the detection of cancer due to the differential expression of miRNAs, such as oncogenic and tumor-suppressor miRNAs, between cancer cells and normal cells.[126] In fact, exosomal miRNA signatures are implicated in prostate cancer, glioblastoma, breast cancer, and gastric cancer, and new signatures are constantly emerging.[127-130] Unsurprisingly, analyses of exosomal protein and lipid composition also showed promising prognostic potential in many different cancers.[131-134] Taken together, the unique components constituted within exosomes during their biogenesis can provide a multi-faceted, combinatorial approach in early detection of cancer. Moreover, insights into specific disease-generating entities gained by studying exosomes may allow for more specific and directed treatments.

Exosome Biogenesis Inhibition

In addition to utilizing exosomes as immunotherapy tools, inhibiting the biogenesis of tumor exosomes in combination with other therapies might provide benefits for cancer treatment. GW4869 is a small-molecule inhibitor for ceramide-facilitated MVBs formation, thus preventing the production of exosomes. Viabilities for GW4869-treated prostate cancer cells are lower in

both normoxia and hypoxia conditions.[135] Pancreatic cancer cells exhibit hypersecretion of exosomes upon chemotherapy treatment, and GW4869 increases the cellular sensitivity towards the gemcitabine.[136] The GW4869 also exhibits the ability to limit lung multiplicities, decreasing cancer metastasis potential in mouse models.[137] Rab27a and Rab27b are important proteins in exosomal secretion pathways, while the cells with Rab27a or Rab27b knockdown significantly reduce exosomal production without disturbing composition.[138] In another study, a metastatic lung cell line 4T1 was first transfected with shRNA that could silence the expression of Rab27a,[139] and the mouse model implanted with the transfected 4T1 cell showed reduced tumor growth and metastasis. Ketoconazole, an FDA-approved drug for prostate cancer, showed dose-dependent inhibition for Rab27a as well as other exosomal secretion related proteins including Alix and nSMase2.[140] Though the current pharmacological mechanism of ketoconazole for prostate cancer is to block androgen synthesis,[141] its effect in exosomal secretion inhibition may add additional anti-cancer benefits. Nevertheless, the exosomes biogenesis plays a critical role in normal physiological functions, so the unselective inhibition might result in severe adverse events.[106] Therefore, to exploit the inhibition of exosome biogenesis for cancer therapy, selective blockade of tumor-derived exosomes or targeted delivery to tumor sites might be a safer choice.

Concluding Remarks

Ever since exosomes were first discovered, meaningful scientific progresses were made to reveal exosomes' potential in cancer immunotherapy. The main challenge in DEXs is to elicit antigen-specific T-cell responses and inhibit immune checkpoint-related immunosuppression. DEXs allow for the delivery of immune checkpoint modulators in a cell-specific manner, thus, improving modulation of the tumor microenvironment. Modification of DEXs with immunostimulatory ligands or co-administration of DEXs with adjuvants might improve the recognition of DEXs by the immune system. On the other hand, the enriched tumor antigens on TEXs' surfaces make them ideal candidates as antigen presenters, but their immune escape mechanism and immunosuppressive ligands and cargos may mitigate their immune-inducing advantages. Importantly, using genetic or nongenetic approaches to increase the expression of immunostimulatory ligands or cargo contents can enhance the overall immune response. Treating TEXs with RNAs or certain small-molecule inhibitors might partially deactivate the immunosuppressive activity. Additionally, both DEXs and TEXs require autologous cell culture or

ascites, so they are unlikely to be immediately available as therapeutic tools that can be produced in large scale. The alternative choice is to use stable cell lines that can be maintained *ex vivo*. Cell-derived exosomes are versatile in editing the targeting or therapeutic ligands on surface or cytoplasmic cargos. To maximize the efficacy, multifunctional exosomes that integrate the targeting, immunostimulatory and drug-carrying functions should be further explored. Lastly, preparation of high-purity exosomes products and isolating a single population of exosomes for characterization are still difficult for current technologies. Developing a more comprehensive understanding of the immunological functions and composition together with enhanced productivity for clinical applications will foster innovations for exosome-based cancer immunotherapy.

Acknowledgements

This work was supported in part by STOP CANCER Research Career Development Award (to Y.Z), Department of Defense CDMRP Career Development Award W81XWH-19-1-0272 (to Y.Z), AACR-Bayer Innovation and Discovery Grant (to Y.Z), California Breast Cancer Research Program Innovative Development and Exploratory Award 25IB-0080 (to Y.Z), and Tobacco Related-Disease Research Program New Investigator Award T30KT1021 (to Y.Z). The figures were created with BioRender.com.

References

1. van Niel, G.; D'Angelo, G.; Raposo, G., Shedding light on the cell biology of extracellular vesicles. *Nature Reviews Molecular Cell Biology* **2018,** *19* (4), 213-228.
2. Deatherage, B. L.; Cookson, B. T., Membrane Vesicle Release in Bacteria, Eukaryotes, and Archaea: a Conserved yet Underappreciated Aspect of Microbial Life. *Infection and Immunity* **2012,** *80* (6), 1948-1957.
3. Johnstone, R. M.; Adam, M.; Hammond, J. R.; Orr, L.; Turbide, C., Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *The Journal of Biological Chemistry* **1987,** *262* (19), 9412-9420.
4. Colombo, M.; Raposo, G.; Théry, C., Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol.* **2014,** *30*, 255-289.
5. Kalluri, R., The biology and function of exosomes in cancer. *The Journal of Clinical Investigation* **2016,** *126* (4), 1208-1215.
6. Mathieu, M.; Martin-Jaular, L.; Lavieu, G.; Théry, C., Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol* **2019,** *21* (1), 9-17.

7. Kalluri, R.; LeBleu, V. S., The biology, function, and biomedical applications of exosomes. *Science* **2020,** *367* (6478).
8. Clotilde, T.; et al., Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles* **2018,** *7* (1), 1535750.
9. Cheng, Q.; Shi, X.; Han, M.; Smbatyan, G.; Lenz, H.-J.; Zhang, Y., Reprogramming Exosomes as Nanoscale Controllers of Cellular Immunity. *J. Am. Chem. Soc.* **2018,** *140* (48), 16413-16417.
10. Taghikhani, A.; Farzaneh, F.; Sharifzad, F.; Mardpour, S.; Ebrahimi, M.; Hassan, Z. M., Engineered Tumor-Derived Extracellular Vesicles: Potentials in Cancer Immunotherapy. *Front Immunol* **2020,** *11*, 221.
11. Kunigelis, K. E.; Graner, M. W., The Dichotomy of Tumor Exosomes (TEX) in Cancer Immunity: Is It All in the ConTEXt? *Vaccines (Basel)* **2015,** *3* (4), 1019-51.
12. Gao, D.; Jiang, L., Exosomes in cancer therapy: a novel experimental strategy. *Am J Cancer Res* **2018,** *8* (11), 2165-2175.
13. Ludwig, N.; Whiteside, T. L., Potential roles of tumor-derived exosomes in angiogenesis. *Expert Opin Ther Targets* **2018,** *22* (5), 409-417.
14. Whiteside, T. L., Chapter Four - Tumor-Derived Exosomes and Their Role in Cancer Progression. In *Advances in Clinical Chemistry*, Makowski, G. S., Ed. Elsevier: 2016; Vol. 74, pp 103-141.
15. Tian, W.; Liu, S.; Li, B., Potential Role of Exosomes in Cancer Metastasis. *Biomed Res Int* **2019,** *2019*, 4649705.
16. Azmi, A. S.; Bao, B.; Sarkar, F. H., Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. *Cancer Metastasis Rev* **2013,** *32* (3-4), 623-42.
17. Huang, T.; Deng, C. X., Current Progresses of Exosomes as Cancer Diagnostic and Prognostic Biomarkers. *Int J Biol Sci* **2019,** *15* (1), 1-11.
18. Pitt, J. M.; Charrier, M.; Viaud, S.; Andre, F.; Besse, B.; Chaput, N.; Zitvogel, L., Dendritic cell-derived exosomes as immunotherapies in the fight against cancer. *J Immunol* **2014,** *193* (3), 1006-11.
19. Pitt, J. M.; André, F.; Amigorena, S.; Soria, J.-C.; Eggermont, A.; Kroemer, G.; Zitvogel, L., Dendritic cell–derived exosomes for cancer therapy. *The Journal of Clinical Investigation* **2016,** *126* (4), 1224-1232.
20. Pitt, J. M.; Andre, F.; Amigorena, S.; Soria, J. C.; Eggermont, A.; Kroemer, G.; Zitvogel, L., Dendritic cell-derived exosomes for cancer therapy. *J Clin Invest* **2016,** *126* (4), 1224-32.
21. Hessvik, N. P.; Llorente, A., Current knowledge on exosome biogenesis and release. *Cell. Mol. Life Sci.* **2018,** *75* (2), 193-208.
22. Palma, J.; Yaddanapudi, S. C.; Pigati, L.; Havens, M. A.; Jeong, S.; Weiner, G. A.; Weimer, K. M. E.; Stern, B.; Hastings, M. L.; Duelli, D. M., MicroRNAs are exported from malignant cells in customized particles. *Nucleic Acids Res.* **2012,** *40* (18), 9125-9138.
23. Yang, C.; Robbins, P. D., The Roles of Tumor-Derived Exosomes in Cancer Pathogenesis. *Clinical and Developmental Immunology* **2011**.

24. van der Vlist, E. J.; Arkesteijn, G. J. A.; van de Lest, C. H. A.; Stoorvogel, W.; Nolte-'t Hoen, E. N. M.; Wauben, M. H. M., CD4+ T cell activation promotes the differential release of distinct populations of nanosized vesicles. *Journal of Extracellular Vesicles* **2012,** *1*.
25. Andreu, Z.; Yáñez-Mó, M., Tetraspanins in Extracellular Vesicle Formation and Function. *Front Immunol* **2014,** *5*.
26. Bebelman, M. P.; Smit, M. J.; Pegtel, D. M.; Baglio, S. R., Biogenesis and function of extracellular vesicles in cancer. *Pharmacology & Therapeutics* **2018,** *188*, 1-11.
27. Johnstone, R. M.; Mathew, A.; Mason, A. B.; Teng, K., Exosome formation during maturation of mammalian and avian reticulocytes: evidence that exosome release is a major route for externalization of obsolete membrane proteins. *J. Cell. Physiol.* **1991,** *147* (1), 27-36.
28. Laulagnier, K.; Grand, D.; Dujardin, A.; Hamdi, S.; Vincent-Schneider, H.; Lankar, D.; Salles, J.-P.; Bonnerot, C.; Perret, B.; Record, M., PLD2 is enriched on exosomes and its activity is correlated to the release of exosomes. *FEBS Letters* **2004,** *572* (1-3), 11-14.
29. Raposo, G.; Nijman, H. W.; Stoorvogel, W.; Liejendekker, R.; Harding, C. V.; Melief, C. J.; Geuze, H. J., B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* **1996,** *183* (3), 1161-1172.
30. Valadi, H.; Ekström, K.; Bossios, A.; Sjöstrand, M.; Lee, J. J.; Lötvall, J. O., Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* **2007,** *9* (6), 654-659.
31. Kamerkar, S.; LeBleu, V. S.; Sugimoto, H.; Yang, S.; Ruivo, C. F.; Melo, S. A.; Lee, J. J.; Kalluri, R., Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* **2017,** *546* (7659), 498-503.
32. Mulcahy, L. A.; Pink, R. C.; Carter, D. R. F., Routes and mechanisms of extracellular vesicle uptake. *Journal of Extracellular Vesicles* **2014,** *3*.
33. Commisso, C.; Davidson, S. M.; Soydaner-Azeloglu, R. G.; Parker, S. J.; Kamphorst, J. J.; Hackett, S.; Grabocka, E.; Nofal, M.; Drebin, J. A.; Thompson, C. B.; Rabinowitz, J. D.; Metallo, C. M.; Heiden, M. G. V.; Bar-Sagi, D., Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. *Nature* **2013,** *497* (7451), 633-637.
34. Tian, T.; Zhu, Y.-L.; Zhou, Y.-Y.; Liang, G.-F.; Wang, Y.-Y.; Hu, F.-H.; Xiao, Z.-D., Exosome Uptake through Clathrin-mediated Endocytosis and Macropinocytosis and Mediating miR-21 Delivery. *J. Biol. Chem.* **2014,** *289* (32), 22258-22267.
35. Pegtel, D. M.; Cosmopoulos, K.; Thorley-Lawson, D. A.; van Eijndhoven, M. A. J.; Hopmans, E. S.; Lindenberg, J. L.; de Gruijl, T. D.; Würdinger, T.; Middeldorp, J. M., Functional delivery of viral miRNAs via exosomes. *Proc. Natl. Acad. Sci. U.S.A.* **2010,** *107* (14), 6328-6333.
36. Zhou, W.; Fong, M. Y.; Min, Y.; Somlo, G.; Liu, L.; Palomares, M. R.; Yu, Y.; Chow, A.; O'Connor, S. T. F.; Chin, A. R.; Yen, Y.; Wang, Y.; Marcusson, E. G.; Chu, P.; Wu, J.; Wu, X.; Li, A. X.; Li, Z.; Gao, H.; Ren,

X.; Boldin, M. P.; Lin, P. C.; Wang, S. E., Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. *Cancer Cell* **2014,** *25* (4), 501-515.
37. Zitvogel, L.; Regnault, A.; Lozier, A.; Wolfers, J.; Flament, C.; Tenza, D.; Ricciardi-Castagnoli, P.; Raposo, G.; Amigorena, S., Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes. *Nature Medicine* **1998,** *4* (5), 594-600.
38. Vincent-Schneider, H.; Stumptner-Cuvelette, P.; Lankar, D.; Pain, S.; Raposo, G.; Benaroch, P.; Bonnerot, C., Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific T cells. *Int. Immunol.* **2002,** *14* (7), 713-722.
39. Tkach, M.; Kowal, J.; Zucchetti, A. E.; Enserink, L.; Jouve, M.; Lankar, D.; Saitakis, M.; Martin-Jaular, L.; Théry, C., Qualitative differences in T-cell activation by dendritic cell-derived extracellular vesicle subtypes. *The EMBO journal* **2017,** *36* (20), 3012-3028.
40. Wahlund, C. J. E.; Güclüler, G.; Hiltbrunner, S.; Veerman, R. E.; Näslund, T. I.; Gabrielsson, S., Exosomes from antigen-pulsed dendritic cells induce stronger antigen-specific immune responses than microvesicles in vivo. *Scientific Reports* **2017,** *7* (1), 17095.
41. Burrello, J.; Monticone, S.; Gai, C.; Gomez, Y.; Kholia, S.; Camussi, G., Stem Cell-Derived Extracellular Vesicles and Immune-Modulation. *Frontiers in Cell and Developmental Biology* **2016,** *4*, 83.
42. Conforti, A.; Scarsella, M.; Starc, N.; Giorda, E.; Biagini, S.; Proia, A.; Carsetti, R.; Locatelli, F.; Bernardo, M. E., Microvescicles derived from mesenchymal stromal cells are not as effective as their cellular counterpart in the ability to modulate immune responses in vitro. *Stem Cells Dev.* **2014,** *23* (21), 2591-2599.
43. Del Fattore, A.; Luciano, R.; Pascucci, L.; Goffredo, B. M.; Giorda, E.; Scapaticci, M.; Fierabracci, A.; Muraca, M., Immunoregulatory Effects of Mesenchymal Stem Cell-Derived Extracellular Vesicles on T Lymphocytes. *Cell Transplant* **2015,** *24* (12), 2615-2627.
44. Gabrusiewicz, K.; Li, X.; Wei, J.; Hashimoto, Y.; Marisetty, A. L.; Ott, M.; Wang, F.; Hawke, D.; Yu, J.; Healy, L. M.; Hossain, A.; Akers, J. C.; Maiti, S. N.; Yamashita, S.; Shimizu, Y.; Dunner, K.; Zal, M. A.; Burks, J. K.; Gumin, J.; Nwajei, F.; Rezavanian, A.; Zhou, S.; Rao, G.; Sawaya, R.; Fuller, G. N.; Huse, J. T.; Antel, J. P.; Li, S.; Cooper, L.; Sulman, E. P.; Chen, C.; Geula, C.; Kalluri, R.; Zal, T.; Heimberger, A. B., Glioblastoma stem cell-derived exosomes induce M2 macrophages and PD-L1 expression on human monocytes. *Oncoimmunology* **2018,** *7* (4), e1412909.
45. Abusamra, A. J.; Zhong, Z.; Zheng, X.; Li, M.; Ichim, T. E.; Chin, J. L.; Min, W.-P., Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis. *Blood Cells Mol. Dis.* **2005,** *35* (2), 169-173.
46. Ding, G.; Zhou, L.; Qian, Y.; Fu, M.; Chen, J.; Chen, J.; Xiang, J.; Wu, Z.; Jiang, G.; Cao, L., Pancreatic cancer-derived exosomes transfer miRNAs

to dendritic cells and inhibit RFXAP expression via miR-212-3p. *Oncotarget* **2015,** *6* (30), 29877-29888.
47. Wolfers, J.; Lozier, A.; Raposo, G.; Regnault, A.; Théry, C.; Masurier, C.; Flament, C.; Pouzieux, S.; Faure, F.; Tursz, T.; Angevin, E.; Amigorena, S.; Zitvogel, L., Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nature Medicine* **2001,** *7* (3), 297-303.
48. Andre, F.; Schartz, N. E. C.; Movassagh, M.; Flament, C.; Pautier, P.; Morice, P.; Pomel, C.; Lhomme, C.; Escudier, B.; Le Chevalier, T.; Tursz, T.; Amigorena, S.; Raposo, G.; Angevin, E.; Zitvogel, L., Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* **2002,** *360* (9329), 295-305.
49. Zitvogel, L.; Regnault, A.; Lozier, A.; Wolfers, J.; Flament, C.; Tenza, D.; Ricciardi-Castagnoli, P.; Raposo, G.; Amigorena, S., Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* **1998,** *4* (5), 594-600.
50. Tian, H.; Li, W., Dendritic cell-derived exosomes for cancer immunotherapy: hope and challenges. *Ann Transl Med* **2017,** *5* (10), 221.
51. Ren, G.; Wang, Y.; Yuan, S.; Wang, B., Dendritic cells loaded with HeLa-derived exosomes simulate an antitumor immune response. *Oncol Lett* **2018,** *15* (5), 6636-6640.
52. Segura, E.; Amigorena, S.; Thery, C., Mature dendritic cells secrete exosomes with strong ability to induce antigen-specific effector immune responses. *Blood Cells Mol Dis* **2005,** *35* (2), 89-93.
53. Muntasell, A.; Berger, A. C.; Roche, P. A., T cell-induced secretion of MHC class II-peptide complexes on B cell exosomes. *EMBO J* **2007,** *26* (19), 4263-72.
54. Vincent-Schneider, H.; Stumptner-Cuvelette, P.; Lankar, D.; Pain, S.; Raposo, G.; Benaroch, P.; Bonnerot, C., Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific T cells. *Int Immunol* **2002,** *14* (7), 713-22.
55. Théry, C.; Duban, L.; Segura, E.; Véron, P.; Lantz, O.; Amigorena, S., Indirect activation of naïve CD4+ T cells by dendritic cell–derived exosomes. *Nature Immunology* **2002,** *3* (12), 1156-1162.
56. Admyre, C.; Johansson, S. M.; Paulie, S.; Gabrielsson, S., Direct exosome stimulation of peripheral human T cells detected by ELISPOT. *Eur J Immunol* **2006,** *36* (7), 1772-81.
57. Robbins, P. D.; Morelli, A. E., Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* **2014,** *14* (3), 195-208.
58. Segura, E.; Nicco, C.; Lombard, B. r. r.; Véron, P.; Raposo, G. a.; Batteux, F. d. r.; Amigorena, S.; Théry, C., ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood* **2005,** *106* (1), 216-223.

59. Thery, C.; Duban, L.; Segura, E.; Veron, P.; Lantz, O.; Amigorena, S., Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat Immunol* **2002,** *3* (12), 1156-62.
60. Hao, S.; Bai, O.; Li, F.; Yuan, J.; Laferte, S.; Xiang, J., Mature dendritic cells pulsed with exosomes stimulate efficient cytotoxic T-lymphocyte responses and antitumour immunity. *Immunology* **2007,** *120* (1), 90-102.
61. Romagnoli, G. G.; Zelante, B. B.; Toniolo, P. A.; Migliori, I. K.; Barbuto, J. A., Dendritic Cell-Derived Exosomes may be a Tool for Cancer Immunotherapy by Converting Tumor Cells into Immunogenic Targets. *Front Immunol* **2014,** *5*, 692.
62. Wu, Q.; Zhou, L.; Lv, D.; Zhu, X.; Tang, H., Exosome-mediated communication in the tumor microenvironment contributes to hepatocellular carcinoma development and progression. *J Hematol Oncol* **2019,** *12* (1), 53.
63. Maia, J.; Caja, S.; Strano Moraes, M. C.; Couto, N.; Costa-Silva, B., Exosome-Based Cell-Cell Communication in the Tumor Microenvironment. *Front Cell Dev Biol* **2018,** *6* (18), 18.
64. Olejarz, W.; Dominiak, A.; Zolnierzak, A.; Kubiak-Tomaszewska, G.; Lorenc, T., Tumor-Derived Exosomes in Immunosuppression and Immunotherapy. *J Immunol Res* **2020,** *2020*, 6272498.
65. Hong, B. S.; Cho, J. H.; Kim, H.; Choi, E. J.; Rho, S.; Kim, J.; Kim, J. H.; Choi, D. S.; Kim, Y. K.; Hwang, D.; Gho, Y. S., Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells. *BMC Genomics* **2009,** *10*, 556.
66. Tung, K. H.; Ernstoff, M. S.; Allen, C.; Shu, S., A Review of Exosomes and their Role in The Tumor Microenvironment and Host-Tumor "Macroenvironment". *J Immunol Sci* **2019,** *3* (1), 4-8.
67. Ribeiro, M. F.; Zhu, H.; Millard, R. W.; Fan, G. C., Exosomes Function in Pro- and Anti-Angiogenesis. *Curr Angiogenes* **2013,** *2* (1), 54-59.
68. Muz, B.; de la Puente, P.; Azab, F.; Azab, A. K., The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia (Auckl)* **2015,** *3*, 83-92.
69. Guo, Y.; Ji, X.; Liu, J.; Fan, D.; Zhou, Q.; Chen, C.; Wang, W.; Wang, G.; Wang, H.; Yuan, W.; Ji, Z.; Sun, Z., Effects of exosomes on pre-metastatic niche formation in tumors. *Mol Cancer* **2019,** *18* (1), 39.
70. Huang, W.; Yan, Y.; Liu, Y.; Lin, M.; Ma, J.; Zhang, W.; Dai, J.; Li, J.; Guo, Q.; Chen, H.; Makabel, B.; Liu, H.; Su, C.; Bi, H.; Zhang, J., Exosomes with low miR-34c-3p expression promote invasion and migration of non-small cell lung cancer by upregulating integrin alpha2beta1. *Signal Transduct Target Ther* **2020,** *5* (1), 39.
71. Wang, B.; Tan, Z.; Guan, F., Tumor-Derived Exosomes Mediate the Instability of Cadherins and Promote Tumor Progression. *Int J Mol Sci* **2019,** *20* (15), 3652.

72. Corcoran, C.; Rani, S.; O'Brien, K.; O'Neill, A.; Prencipe, M.; Sheikh, R.; Webb, G.; McDermott, R.; Watson, W.; Crown, J.; O'Driscoll, L., Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes. *PLoS One* **2012,** *7* (12), e50999.
73. Liu, C.; Yu, S.; Zinn, K.; Wang, J.; Zhang, L.; Jia, Y.; Kappes, J. C.; Barnes, S.; Kimberly, R. P.; Grizzle, W. E.; Zhang, H. G., Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function. *J Immunol* **2006,** *176* (3), 1375-85.
74. Clayton A, M. J., Court J, Mason MD, Tabi Z, Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. *Cancer Research* **2007,** *67* (15), 7458-7466.
75. Maybruck, B. T.; Pfannenstiel, L. W.; Diaz-Montero, M.; Gastman, B. R., Tumor-derived exosomes induce CD8(+) T cell suppressors. *J Immunother Cancer* **2017,** *5* (1), 65.
76. Tian, X.; Shen, H.; Li, Z.; Wang, T.; Wang, S., Tumor-derived exosomes, myeloid-derived suppressor cells, and tumor microenvironment. *J Hematol Oncol* **2019,** *12* (1), 84.
77. Bu, N.; Li, Q. L.; Feng, Q.; Sun, B. Z., Immune protection effect of exosomes against attack of L1210 tumor cells. *Leuk Lymphoma* **2006,** *47* (5), 913-8.
78. Graner, M. W.; Alzate, O.; Dechkovskaia, A. M.; Keene, J. D.; Sampson, J. H.; Mitchell, D. A.; Bigner, D. D., Proteomic and immunologic analyses of brain tumor exosomes. *FASEB J* **2009,** *23* (5), 1541-57.
79. Hickman-Miller, H. D.; Hildebrand, W. H., The immune response under stress: the role of HSP-derived peptides. *Trends Immunol* **2004,** *25* (8), 427-33.
80. Murshid, A.; Gong, J.; Calderwood, S. K., The role of heat shock proteins in antigen cross presentation. *Front Immunol* **2012,** *3*, 63.
81. Cho, J. A.; Lee, Y. S.; Kim, S. H.; Ko, J. K.; Kim, C. W., MHC independent anti-tumor immune responses induced by Hsp70-enriched exosomes generate tumor regression in murine models. *Cancer Lett* **2009,** *275* (2), 256-65.
82. Xie, Y.; Bai, O.; Zhang, H.; Yuan, J.; Zong, S.; Chibbar, R.; Slattery, K.; Qureshi, M.; Wei, Y.; Deng, Y.; Xiang, J., Membrane-bound HSP70-engineered myeloma cell-derived exosomes stimulate more efficient CD8(+) CTL- and NK-mediated antitumour immunity than exosomes released from heat-shocked tumour cells expressing cytoplasmic HSP70. *J Cell Mol Med* **2010,** *14* (11), 2655-66.
83. Lv, L. H.; Wan, Y. L.; Lin, Y.; Zhang, W.; Yang, M.; Li, G. L.; Lin, H. M.; Shang, C. Z.; Chen, Y. J.; Min, J., Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro. *J Biol Chem* **2012,** *287* (19), 15874-85.

84. Cho, J. A.; Yeo, D. J.; Son, H. Y.; Kim, H. W.; Jung, D. S.; Ko, J. K.; Koh, J. S.; Kim, Y. N.; Kim, C. W., Exosomes: a new delivery system for tumor antigens in cancer immunotherapy. *Int J Cancer* **2005,** *114* (4), 613-22.
85. Rivoltini, L.; Chiodoni, C.; Squarcina, P.; Tortoreto, M.; Villa, A.; Vergani, B.; Burdek, M.; Botti, L.; Arioli, I.; Cova, A.; Mauri, G.; Vergani, E.; Bianchi, B.; Della Mina, P.; Cantone, L.; Bollati, V.; Zaffaroni, N.; Gianni, A. M.; Colombo, M. P.; Huber, V., TNF-Related Apoptosis-Inducing Ligand (TRAIL)-Armed Exosomes Deliver Proapoptotic Signals to Tumor Site. *Clin Cancer Res* **2016,** *22* (14), 3499-512.
86. Munoz, J. L.; Bliss, S. A.; Greco, S. J.; Ramkissoon, S. H.; Ligon, K. L.; Rameshwar, P., Delivery of Functional Anti-miR-9 by Mesenchymal Stem Cell-derived Exosomes to Glioblastoma Multiforme Cells Conferred Chemosensitivity. *Mol Ther Nucleic Acids* **2013,** *2* (10), e126.
87. O'Brien, K.; Lowry, M. C.; Corcoran, C.; Martinez, V. G.; Daly, M.; Rani, S.; Gallagher, W. M.; Radomski, M. W.; MacLeod, R. A.; O'Driscoll, L., miR-134 in extracellular vesicles reduces triple-negative breast cancer aggression and increases drug sensitivity. *Oncotarget* **2015,** *6* (32), 32774-89.
88. Gu, X.; Erb, U.; Buchler, M. W.; Zoller, M., Improved vaccine efficacy of tumor exosome compared to tumor lysate loaded dendritic cells in mice. *Int. J. Cancer* **2015,** *136* (4), E74-84.
89. Luan, X.; Sansanaphongpricha, K.; Myers, I.; Chen, H.; Yuan, H.; Sun, D., Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta pharmacologica Sinica* **2017,** *38* (6), 754-763.
90. Dai, S.; Zhou, X.; Wang, B.; Wang, Q.; Fu, Y.; Chen, T.; Wan, T.; Yu, Y.; Cao, X., Enhanced induction of dendritic cell maturation and HLA-A*0201-restricted CEA-specific CD8(+) CTL response by exosomes derived from IL-18 gene-modified CEA-positive tumor cells. *J Mol Med (Berl)* **2006,** *84* (12), 1067-1076.
91. Yang, Y.; Xiu, F.; Cai, Z.; Wang, J.; Wang, Q.; Fu, Y.; Cao, X., Increased induction of antitumor response by exosomes derived from interleukin-2 gene-modified tumor cells. *J Cancer Res Clin Oncol* **2007,** *133* (6), 389-99.
92. Andre, F.; Chaput, N.; Schartz, N. E.; Flament, C.; Aubert, N.; Bernard, J.; Lemonnier, F.; Raposo, G.; Escudier, B.; Hsu, D. H.; Tursz, T.; Amigorena, S.; Angevin, E.; Zitvogel, L., Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. *J Immunol* **2004,** *172* (4), 2126-36.
93. Ye, Z.; Zhang, T.; He, W.; Jin, H.; Liu, C.; Yang, Z.; Ren, J., Methotrexate-Loaded Extracellular Vesicles Functionalized with Therapeutic and Targeted Peptides for the Treatment of Glioblastoma Multiforme. *ACS Appl Mater Interfaces* **2018,** *10* (15), 12341-12350.
94. Xiu, F.; Cai, Z.; Yang, Y.; Wang, X.; Wang, J.; Cao, X., Surface anchorage of superantigen SEA promotes induction of specific antitumor immune

response by tumor-derived exosomes. *J Mol Med (Berl)* **2007,** *85* (5), 511-21.
95. Xiaojing Shi , Q. C., Yong Zhang Reprogramming Extracellular Vesicles With Engineered Proteins. *Methods .* **2019,** *177*, 95-102.
96. Shi, X.; Cheng, Q.; Hou, T.; Han, M.; Smbatyan, G.; Lang, J. E.; Epstein, A. L.; Lenz, H. J.; Zhang, Y., Genetically Engineered Cell-Derived Nanoparticles for Targeted Breast Cancer Immunotherapy. *Molecular therapy : the journal of the American Society of Gene Therapy* **2020,** *28* (2), 536-547.
97. Qinqin Cheng 1 , X. S., Menglu Han 1 , Goar Smbatyan 2 , Heinz-Josef Lenz 2 , Yong Zhang Reprogramming Exosomes as Nanoscale Controllers of Cellular Immunity. *J Am Chem Soc.* **2018,** *140* (48), 16413-16417.
98. Cheng, Q.; Shi, X.; Zhang, Y., Reprogramming Exosomes for Immunotherapy. *Methods Mol. Biol.* **2020,** *2097*, 197-209.
99. Skog, J.; Würdinger, T.; van Rijn, S.; Meijer, D. H.; Gainche, L.; Sena-Esteves, M.; Curry, W. T., Jr.; Carter, B. S.; Krichevsky, A. M.; Breakefield, X. O., Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* **2008,** *10* (12), 1470-1476.
100. Sun, Z.; Shi, K.; Yang, S.; Liu, J.; Zhou, Q.; Wang, G.; Song, J.; Li, Z.; Zhang, Z.; Yuan, W., Effect of exosomal miRNA on cancer biology and clinical applications. *Mol Cancer* **2018,** *17* (1), 147.
101. Zheng, R.; Du, M.; Wang, X.; Xu, W.; Liang, J.; Wang, W.; Lv, Q.; Qin, C.; Chu, H.; Wang, M.; Yuan, L.; Qian, J.; Zhang, Z., Exosome-transmitted long non-coding RNA PTENP1 suppresses bladder cancer progression. *Mol Cancer* **2018,** *17* (1), 143.
102. Zhou, R.; Chen, K. K.; Zhang, J.; Xiao, B.; Huang, Z.; Ju, C.; Sun, J.; Zhang, F.; Lv, X. B.; Huang, G., The decade of exosomal long RNA species: an emerging cancer antagonist. *Mol Cancer* **2018,** *17* (1), 75.
103. Yang, T.; Fogarty, B.; LaForge, B.; Aziz, S.; Pham, T.; Lai, L.; Bai, S., Delivery of Small Interfering RNA to Inhibit Vascular Endothelial Growth Factor in Zebrafish Using Natural Brain Endothelia Cell-Secreted Exosome Nanovesicles for the Treatment of Brain Cancer. *Aaps j* **2017,** *19* (2), 475-486.
104. Yang, Z.; Xie, J.; Zhu, J.; Kang, C.; Chiang, C.; Wang, X.; Wang, X.; Kuang, T.; Chen, F.; Chen, Z.; Zhang, A.; Yu, B.; Lee, R. J.; Teng, L.; Lee, L. J., Functional exosome-mimic for delivery of siRNA to cancer: in vitro and in vivo evaluation. *J Control Release* **2016,** *243*, 160-171.
105. Kranz, L. M.; Diken, M.; Haas, H.; Kreiter, S.; Loquai, C.; Reuter, K. C.; Meng, M.; Fritz, D.; Vascotto, F.; Hefesha, H.; Grunwitz, C.; Vormehr, M.; Husemann, Y.; Selmi, A.; Kuhn, A. N.; Buck, J.; Derhovanessian, E.; Rae, R.; Attig, S.; Diekmann, J.; Jabulowsky, R. A.; Heesch, S.; Hassel, J.; Langguth, P.; Grabbe, S.; Huber, C.; Tureci, O.; Sahin, U., Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. *Nature* **2016,** *534* (7607), 396-401.

106. Batista, I. A.; Melo, S. A., Exosomes and the Future of Immunotherapy in Pancreatic Cancer. *Int J Mol Sci* **2019,** *20* (3), 567.
107. Johnstone, R. M.; Adam, M.; Pan, B. T., The fate of the transferrin receptor during maturation of sheep reticulocytes in vitro. *Can J Biochem Cell Biol* **1984,** *62* (11), 1246-54.
108. van Renswoude, J.; Bridges, K. R.; Harford, J. B.; Klausner, R. D., Receptor-mediated endocytosis of transferrin and the uptake of fe in K562 cells: identification of a nonlysosomal acidic compartment. *Proc Natl Acad Sci U S A* **1982,** *79* (20), 6186-90.
109. Morse, M. A.; Garst, J.; Osada, T.; Khan, S.; Hobeika, A.; Clay, T. M.; Valente, N.; Shreeniwas, R.; Sutton, M. A.; Delcayre, A.; Hsu, D. H.; Le Pecq, J. B.; Lyerly, H. K., A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J Transl Med* **2005,** *3* (1), 9.
110. Escudier, B.; Dorval, T.; Chaput, N.; Andre, F.; Caby, M. P.; Novault, S.; Flament, C.; Leboulaire, C.; Borg, C.; Amigorena, S.; Boccaccio, C.; Bonnerot, C.; Dhellin, O.; Movassagh, M.; Piperno, S.; Robert, C.; Serra, V.; Valente, N.; Le Pecq, J. B.; Spatz, A.; Lantz, O.; Tursz, T.; Angevin, E.; Zitvogel, L., Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. *J Transl Med* **2005,** *3* (1), 10.
111. Ratajczak, J.; Miekus, K.; Kucia, M.; Zhang, J.; Reca, R.; Dvorak, P.; Ratajczak, M. Z., Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* **2006,** *20* (5), 847-56.
112. Dai, S.; Wei, D.; Wu, Z.; Zhou, X.; Wei, X.; Huang, H.; Li, G., Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. *Mol Ther* **2008,** *16* (4), 782-90.
113. Besse, B.; Charrier, M.; Lapierre, V.; Dansin, E.; Lantz, O.; Planchard, D.; Le Chevalier, T.; Livartoski, A.; Barlesi, F.; Laplanche, A.; Ploix, S.; Vimond, N.; Peguillet, I.; Théry, C.; Lacroix, L.; Zoernig, I.; Dhodapkar, K.; Dhodapkar, M.; Viaud, S.; Soria, J.-C.; Reiners, K. S.; Pogge von Strandmann, E.; Vély, F.; Rusakiewicz, S.; Eggermont, A.; Pitt, J. M.; Zitvogel, L.; Chaput, N., Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC. *Oncoimmunology* **2015,** *5* (4), e1071008-e1071008.
114. Colao, I. L.; Corteling, R.; Bracewell, D.; Wall, I., Manufacturing Exosomes: A Promising Therapeutic Platform. *Trends Mol Med* **2018,** *24* (3), 242-256.
115. Li, K.; Wong, D. K.; Hong, K. Y.; Raffai, R. L., Cushioned-Density Gradient Ultracentrifugation (C-DGUC): A Refined and High Performance Method for the Isolation, Characterization, and Use of Exosomes. *Methods Mol Biol* **2018,** *1740*, 69-83.
116. Gupta, S.; Rawat, S.; Arora, V.; Kottarath, S. K.; Dinda, A. K.; Vaishnav, P. K.; Nayak, B.; Mohanty, S., An improvised one-step sucrose cushion ultracentrifugation method for exosome isolation from culture

supernatants of mesenchymal stem cells. *Stem Cell Res Ther* **2018,** *9* (1), 180.
117. Onodi, Z.; Pelyhe, C.; Terezia Nagy, C.; Brenner, G. B.; Almasi, L.; Kittel, A.; Mancek-Keber, M.; Ferdinandy, P.; Buzas, E. I.; Giricz, Z., Isolation of High-Purity Extracellular Vesicles by the Combination of Iodixanol Density Gradient Ultracentrifugation and Bind-Elute Chromatography From Blood Plasma. *Front Physiol* **2018,** *9*, 1479.
118. Li-Li Yu, J. Z., Jin-Xia Liu, Feng Jiang, Wen-Kai Ni, Li-Shuai Qu, Run-Zhou Ni, Cui-Hua Lu , and Ming-Bing Xiao A Comparison of Traditional and Novel Methods for the Separation of Exosomes from Human Samples. *Biomed Res Int* **2018,** *2018*, 1-9.
119. Busatto, S.; Vilanilam, G.; Ticer, T.; Lin, W. L.; Dickson, D. W.; Shapiro, S.; Bergese, P.; Wolfram, J., Tangential Flow Filtration for Highly Efficient Concentration of Extracellular Vesicles from Large Volumes of Fluid. *Cells* **2018,** *7* (12), 273.
120. Greening, D. W.; Xu, R.; Ji, H.; Tauro, B. J.; Simpson, R. J., A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Methods Mol Biol* **2015,** *1295*, 179-209.
121. Ludwig, A. K.; De Miroschedji, K.; Doeppner, T. R.; Borger, V.; Ruesing, J.; Rebmann, V.; Durst, S.; Jansen, S.; Bremer, M.; Behrmann, E.; Singer, B. B.; Jastrow, H.; Kuhlmann, J. D.; El Magraoui, F.; Meyer, H. E.; Hermann, D. M.; Opalka, B.; Raunser, S.; Epple, M.; Horn, P. A.; Giebel, B., Precipitation with polyethylene glycol followed by washing and pelleting by ultracentrifugation enriches extracellular vesicles from tissue culture supernatants in small and large scales. *J Extracell Vesicles* **2018,** *7* (1), 1528109.
122. Thakur, B. K.; Zhang, H.; Becker, A.; Matei, I.; Huang, Y.; Costa-Silva, B.; Zheng, Y.; Hoshino, A.; Brazier, H.; Xiang, J.; Williams, C.; Rodriguez-Barrueco, R.; Silva, J. M.; Zhang, W.; Hearn, S.; Elemento, O.; Paknejad, N.; Manova-Todorova, K.; Welte, K.; Bromberg, J.; Peinado, H.; Lyden, D., Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Research* **2014,** *24* (6), 766-769.
123. Kahlert, C.; Melo, S. A.; Protopopov, A.; Tang, J.; Seth, S.; Koch, M.; Zhang, J.; Weitz, J.; Chin, L.; Futreal, A.; Kalluri, R., Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. *The Journal of Biological Chemistry* **2014,** *289* (7), 3869-3875.
124. Allenson, K.; Castillo, J.; San Lucas, F. A.; Scelo, G.; Kim, D. U.; Bernard, V.; Davis, G.; Kumar, T.; Katz, M.; Overman, M. J.; Foretova, L.; Fabianova, E.; Holcatova, I.; Janout, V.; Meric-Bernstam, F.; Gascoyne, P.; Wistuba, I.; Varadhachary, G.; Brennan, P.; Hanash, S.; Li, D.; Maitra, A.; Alvarez, H., High prevalence of mutant KRAS in circulating exosome-derived DNA from early-stage pancreatic cancer patients. *Annals of Oncology* **2017,** *28* (4), 741-747.

125. Yang, S.; Che, S. P. Y.; Kurywchak, P.; Tavormina, J. L.; Gansmo, L. B.; Correa de Sampaio, P.; Tachezy, M.; Bockhorn, M.; Gebauer, F.; Haltom, A. R.; Melo, S. A.; LeBleu, V. S.; Kalluri, R., Detection of mutant KRAS and TP53 DNA in circulating exosomes from healthy individuals and patients with pancreatic cancer. *Cancer Biology & Therapy* **2017,** *18* (3), 158-165.
126. Salehi, M.; Sharifi, M., Exosomal miRNAs as novel cancer biomarkers: Challenges and opportunities. *Journal of Cellular Physiology* **2018,** *233* (9), 6370-6380.
127. Alhasan, A. H.; Scott, A. W.; Wu, J. J.; Feng, G.; Meeks, J. J.; Thaxton, C. S.; Mirkin, C. A., Circulating microRNA signature for the diagnosis of very high-risk prostate cancer. *Proceedings of the National Academy of Sciences* **2016,** *113* (38), 10655-10660.
128. Ebrahimkhani, S.; Vafaee, F.; Hallal, S.; Wei, H.; Lee, M. Y. T.; Young, P. E.; Satgunaseelan, L.; Beadnall, H.; Barnett, M. H.; Shivalingam, B.; Suter, C. M.; Buckland, M. E.; Kaufman, K. L., Deep sequencing of circulating exosomal microRNA allows non-invasive glioblastoma diagnosis. *npj Precision Oncology* **2018,** *2* (1), 1-9.
129. Halvaei, S.; Daryani, S.; Eslami-S, Z.; Samadi, T.; Jafarbeik-Iravani, N.; Bakhshayesh, T. O.; Majidzadeh-A, K.; Esmaeili, R., Exosomes in Cancer Liquid Biopsy: A Focus on Breast Cancer. *Molecular Therapy - Nucleic Acids* **2018,** *10*, 131-141.
130. Zhou, X.; Zhu, W.; Li, H.; Wen, W.; Cheng, W.; Wang, F.; Wu, Y.; Qi, L.; Fan, Y.; Chen, Y.; Ding, Y.; Xu, J.; Qian, J.; Huang, Z.; Wang, T.; Zhu, D.; Shu, Y.; Liu, P., Diagnostic value of a plasma microRNA signature in gastric cancer: a microRNA expression analysis. *Scientific Reports* **2015,** *5*.
131. Chen, I. H.; Xue, L.; Hsu, C.-C.; Paez, J. S. P.; Pan, L.; Andaluz, H.; Wendt, M. K.; Iliuk, A. B.; Zhu, J.-K.; Tao, W. A., Phosphoproteins in extracellular vesicles as candidate markers for breast cancer. *Proceedings of the National Academy of Sciences of the United States of America* **2017,** *114* (12), 3175-3180.
132. Castillo, J.; Bernard, V.; San Lucas, F. A.; Allenson, K.; Capello, M.; Kim, D. U.; Gascoyne, P.; Mulu, F. C.; Stephens, B. M.; Huang, J.; Wang, H.; Momin, A. A.; Jacamo, R. O.; Katz, M.; Wolff, R.; Javle, M.; Varadhachary, G.; Wistuba, I. I.; Hanash, S.; Maitra, A.; Alvarez, H., Surfaceome profiling enables isolation of cancer-specific exosomal cargo in liquid biopsies from pancreatic cancer patients. *Annals of Oncology: Official Journal of the European Society for Medical Oncology* **2018,** *29* (1), 223-229.
133. Lai, X.; Wang, M.; McElyea, S. D.; Sherman, S.; House, M.; Korc, M., A microRNA signature in circulating exosomes is superior to exosomal glypican-1 levels for diagnosing pancreatic cancer. *Cancer Letters* **2017,** *393*, 86-93.

134. Sharma, R.; Huang, X.; Brekken, R. A.; Schroit, A. J., Detection of phosphatidylserine-positive exosomes for the diagnosis of early-stage malignancies. *British Journal of Cancer* **2017,** *117* (4), 545-552.
135. Panigrahi, G. K.; Praharaj, P. P.; Peak, T. C.; Long, J.; Singh, R.; Rhim, J. S.; Abd Elmageed, Z. Y.; Deep, G., Hypoxia-induced exosome secretion promotes survival of African-American and Caucasian prostate cancer cells. *Sci Rep* **2018,** *8* (1), 3853.
136. Richards, K. E.; Zeleniak, A. E.; Fishel, M. L.; Wu, J.; Littlepage, L. E.; Hill, R., Cancer-associated fibroblast exosomes regulate survival and proliferation of pancreatic cancer cells. *Oncogene* **2017,** *36* (13), 1770-1778.
137. Fabbri, M.; Paone, A.; Calore, F.; Galli, R.; Gaudio, E.; Santhanam, R.; Lovat, F.; Fadda, P.; Mao, C.; Nuovo, G. J.; Zanesi, N.; Crawford, M.; Ozer, G. H.; Wernicke, D.; Alder, H.; Caligiuri, M. A.; Nana-Sinkam, P.; Perrotti, D.; Croce, C. M., MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc Natl Acad Sci U S A* **2012,** *109* (31), E2110-6.
138. Ostrowski, M.; Carmo, N. B.; Krumeich, S.; Fanget, I.; Raposo, G.; Savina, A.; Moita, C. F.; Schauer, K.; Hume, A. N.; Freitas, R. P.; Goud, B.; Benaroch, P.; Hacohen, N.; Fukuda, M.; Desnos, C.; Seabra, M. C.; Darchen, F.; Amigorena, S.; Moita, L. F.; Thery, C., Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol* **2010,** *12* (1), 19-30; sup pp 1-13.
139. Bobrie, A.; Krumeich, S.; Reyal, F.; Recchi, C.; Moita, L. F.; Seabra, M. C.; Ostrowski, M.; Thery, C., Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression. *Cancer Res* **2012,** *72* (19), 4920-30.
140. Datta, A.; Kim, H.; McGee, L.; Johnson, A. E.; Talwar, S.; Marugan, J.; Southall, N.; Hu, X.; Lal, M.; Mondal, D.; Ferrer, M.; Abdel-Mageed, A. B., High-throughput screening identified selective inhibitors of exosome biogenesis and secretion: A drug repurposing strategy for advanced cancer. *Sci Rep* **2018,** *8* (1), 8161.
141. Pont, A.; Williams, P. L.; Azhar, S.; Reitz, R. E.; Bochra, C.; Smith, E. R.; Stevens, D. A., Ketoconazole blocks testosterone synthesis. *Arch Intern Med* **1982,** *142* (12), 2137-40.