The Role of Extracellular Vesicles in Cancer

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Abstract

Intercellular communication is a key feature of cancer progression and metastasis. Extracellular vesicles (EVs) are generated by all cells, including cancer cells and recent studies have identified EVs as key mediators of cell-cell communication via packaging and transfer of bioactive constituents to impact the biology and function of cancer cells and cells of the tumor microenvironment. Here, we review recent advances in understanding the functional contribution of EVs to cancer progression and metastasis, as cancer biomarkers, and the development of cancer therapeutics.

Introduction

Cancer initiation and progression is facilitated by communication between emerging pre-neoplastic/malignant cells and other cells within the tumor, along with host cells within the local tissue and the entire body. Intercellular communication can facilitate microenvironment changes to influence tumor growth and dissemination of cancer cells. Such signaling can occur through secretion of soluble factors or exchange of extracellular vesicles (EVs). EV secretion was initially described in reticulocytes and was postulated to be a mechanism for removal of excess membrane proteins [1,2]. Additional studies revealed that EVs contain bioactive cargo including proteins, lipids, metabolites, RNA, and DNA that can potentially be transferred to recipient cells to impact their function providing evidence that EVs may act as mediators of intercellular communication. Bidirectional communication mediated by EVs has been identified between numerous cell types within the primary and metastatic tumor microenvironment. EVs have pleotropic roles in processes critical for cancer progression, potentially reflective of their heterogeneous origins and constituents. In addition, the accumulation of EVs in tumors, EV biocompatibility and the ability to readily modify EV cargo have been exploited to develop novel EV based therapeutics that target multiple

aspects of the tumor microenvironment for therapeutic benefit. In this review, we summarize current knowledge of the function of EVs in cancer initiation, progression, metastasis, and response to therapy, as biomarkers, and in the development cancer therapeutics.

The biology and biogenesis of EVs

Extracellular vesicles (EVs) consist of two major subsets: exosomes and ectosomes or microvesicles (Figure 1). Exosomes are generally in the size range of 40–150 nm and ectosomes can be in the size range of 50–1000 nm (Figure 2). The tetraspanins CD9, CD63, and CD81, syntenin, integrins, Alix, TSG101, and flotillin are enriched in EVs, although heterogeneity in expression of some EV biomarkers across cell types is observed [3] and overlap in the size and protein expression between ectosomes and exosomes exists. Ectosomes arise from budding at the plasma membrane and are thought to be enriched for CD9 and CD81 [4,5] and exosomes are enriched in CD63, CD9, CD81, Alix and syntenin. Isolation of pure populations of ectosomes or exosomes has proved challenging due to overlapping protein marker expression and size. Exosomes are derived from the endocytic pathway, wherein budding of late endosomes leads to formation of intraluminal vesicles within multivesicular bodies (MVBs) that contain biomolecules, including protein, RNA, DNA, lipids, and metabolites [6]. The content of EVs is thought to be largely reflective of the cell of origin. Specifically, the metabolic state of the cell can impact EV protein cargo [7], circadian rhythms regulate the packaging of protein into EVs [8], and EVs contain cell type-specific cargo [3]. During MVB formation, several biomolecules are incorporated including RNA and protein. Recent models of exosome release suggest that endoplasmic reticulum (ER)-late endosome membrane contact sites regulate late endosome motility, maturation, and association with small GTPases, ultimately impacting the fusion of MVBs with the plasma membrane and release of exosomes [9]. Specific RNAs are enriched in EVs compared to their cell of origin, suggesting that selective RNA packaging mechanisms control the RNAs loaded into EVs (Figure 1).

The endosomal protein sorting complex (ESCRT) recognizes ubiquitinated cargo and mediates its packaging into MVBs [10]. ESCRT components also control MVB size and the protein cargo of secreted exosomes [11], albeit MVB and exosome biogenesis can occur independent of ESCRTs [12]. Exosomes are enriched with tetraspanins, including CD9, CD63, and CD81. CD63 [13] as well as tetraspanin-enriched microdomains promote the packaging of proteins into exosomes [14]. Despite the identification of several mediators of RNA and protein packaging into EVs, the impact of transferred biomolecules on recipient cell behavior is still unclear. Current approaches rely on silencing miRNAs or genes in the EV producing cell, which may have unintended off-target effects on recipient cell behavior. Alternative strategies that target specific RNA and/or protein packaging molecules or incorporate strategies to inhibit components of EVs without impacting other aspects of signaling in the EV producing cell could further clarify this point.

In addition to RNA and proteins, ssDNA, mtDNA, and dsDNA molecules are present in EVs. DNA packaging into EVs was proposed as a mechanism to remove inflammatory cytoplasmic DNA from cells [15,16]; however, conflicting reports exist on whether DNA is associated with exosomes or small EVs despite employing similar EV isolation techniques

[17,18], suggesting that DNA packaging in EVs may be cell type dependent or that DNA is in low abundance in EVs, limiting its detection. Nonetheless, other studies propose that nuclear content including dsDNA can be packaged into EVs through micronuclei [19]. Alternatively, FLAP/5-lipoxygenase[+] EVs can arise at the nuclear envelope through nSMase1-dependent ceramide synthesis [20].

MVBs that fuse to the plasma membrane release exosomes into the extracellular space, which can then be transferred to recipient cells and potentially impact their function. Rab GTPases regulate vesicle budding and motility to facilitate the trafficking of MVBs for exosome release (Figure 1) [21]. The endosomal pathway is also linked to autophagy as MVBs can fuse with autophagosomes for lysosomal degradation, indicating that autophagy mediators also function in exosome secretion. At the plasma membrane, cortactin in conjunction with Rab27a facilitates MVB docking and exosome release [22] and the composition of the glycocalyx can drive plasma membrane instabilities to facilitate EV secretion [23].

Other nonvesicular nanoparticles that are secreted by cells have also been identified, including exomeres and supermeres. While the precise mechanisms regulating the secretion of exomeres and supermeres remain unknown and whether they are just an aggregated collection of proteins need to be clarified. Exomeres and supermeres appear to be different from small EVs or exosomes based on their size (~45 nm and ~35 nm, respectively). Exomeres are reported to have distinct proteomic profiles and biodistribution patterns compared to small EVs [24], whereas supermeres are enriched with RNAs and have increased accumulation in tissues compared to exomeres and small EVs [25]. Exomeres from MDCK cells are associated with amphiregulin (AREG), which regulates EGFR trafficking in intestinal organoids [26]. Supermeres from colorectal cancer cells are reported to impact lactate secretion and can transfer cetuximab resistance to non-resistant cells [25]. Additional insight into the cargo and physiological functions of extracellular particles is likely to be gained as the biogenesis and biology of such particles is unraveled. Moreover, the majority of studies to understand EV function employ ex vivo isolated EVs and/or bolus administration of EVs. As a result, the physiological role of EV exchange in vivo remains largely unknown and new models that enable fate mapping and tracking endogenous EV release (discussed in more detail in the perspectives and future directions section) and the discovery of more specific mediators of EV secretion will further clarify EV function.

The function of EVs in development

Intercellular communication across cells and tissues is required for proper tissue patterning and development, and many developmental processes are activated in the context of cancer to promote progression. Blastocysts secrete dsDNA-containing EVs prior to implantation providing a potential non-invasive strategy for monitoring embryos [27], but the functional relevance of DNA in blastocyst EVs and in EVs produced by other cell types remains unknown. EV release is considered to be important for maintaining ESC pluripotency via FAK activation [28], which may be a conserved mechanism of stemness maintenance in embryonic stem cells and adult stem cells, including cancer stem-like cells. Incubation of sperm with EVs derived from stressed epididymal epithelial cells led to offspring with

changes in expression of genes related to neurodevelopment and alterations in response to chronic stress [29], suggesting that EVs can transmit information across generations. EV associated dsDNA has 5'-cytosine methylation [30] and proteins identified in histone modification have been identified in EVs [3], raising the possibility that EVs can alter the epigenetic landscape of recipient cells to rewire recipient cell transcription in a more permanent manner. Epigenetic modifications of tumor microenvironment (TME) cells are postulated to play an important role in rewiring TME cell function to promote cancer progression and therapy resistance [31,32]. While it is appreciated that EVs are exchanged in the context of development and can act as morphogens [33–35], the regulatory mechanisms that prevent widespread, non-discriminant EV exchange and allow for specific patterning of organs remain to be unraveled. One possibility is that mechanisms limiting the entry of EVs into cells exist, as demonstrated in the context of lung metastasis [36]. There is also evidence that internalized EVs can be re-released into the extracellular space [37], which may limit the functional impact of EVs. Alternatively, turnover of delivered EV cargo through degradative mechanisms could lead to transient effects on recipient cells. A better understanding of the fate of EVs and their cargo after internalization will clarify their role in eliciting transient vs. long-term effects in the context of normal physiology and cancer.

EV mediated control of aging and metabolism

Cancer is considered to be a disease of aging, as cancer incidence is higher in older individuals in part due to age-dependent accumulation of somatic mutations, but also a result of mutation-independent mechanisms such as increased inflammation and remodeling of the microenvironment [38]. A number of EV-based strategies have been developed to reverse aging phenotypes in vivo. Neonatal umbilical cord mesenchymal stem cell (MSC) derived EVs transfer proliferating cell nuclear antigen (PCNA) to adult bone marrow MSCs and inhibited bone and kidney degeneration associated with aging [39]. EVs from young fibroblasts contain GSTM2 which is transferred to aging tissue to increase GSH levels and reduce ROS and lipid peroxidation [40]. Thus, EVs may have promise as anti-aging agents that could be repurposed for cancer applications, but a better appreciation of the mediators of EV function in aging and overlapping functions in cancer will provide optimal ways to leverage EVs therapeutically.

Communication between organs shapes the overall metabolic state of organisms. Analysis of EVs from distinct cellular sources revealed tissue-specific proteins, providing potential biomarkers of altered tissue metabolism [41]. miRNAs in adipose tissue derived EVs are transferred to the brain to induce damage to synapses and cognitive impairment [42]. In adipose tissue, EVs are exchanged between adipocytes and endothelial cells, enabling the transfer of proteins from endothelial cells to adipocytes. Such transfer is regulated by the systemic nutrient state, with fasting increasing endothelial cell EV secretion [43] and exercise increasing the proteome of EVs in circulation [44]. While these studies have unraveled the role of EVs in the context of altered metabolic states, the function of EVs in establishing and maintaining metabolic homeostasis remains elusive. Endogenous EV transfer between the brain and pancreas has been reported [42], suggesting that EVs may function in hormone regulation in the context of normal physiology. Moreover, EVs have intrinsic metabolic activity [45], suggesting that they have the capacity to remodel local metabolite abundance.

Such EV mediated control of organismal metabolism could have important undiscovered implications in the context of cancer, specifically to mediate metastasis, impact therapeutic responses, and reshape the microbiome.

The impact of EVs on tissue repair, response to stress, and immunity

In the context of damaged tissues and tumors, cells are exposed to many types of stress, including genetic defects, nutrient scarcity, hypoxia, and mechanical stress. Cellular responses to such stresses are pleotropic and context dependent and the same is likely true for EVs. Indeed, EVs have been implicated in facilitating tissue repair and response to stress [46–48]. EVs can have both tissue regenerative and destructive properties, and a comprehensive understanding of their function in response to tissue damage and in mediating tissue repair may provide ways to exploit and/or target EV transfer therapeutically.

Cell-cell signaling is critical for eliciting effective immune responses while preventing overexuberant immune activation that can lead to chronic inflammation and autoimmunity which are risk factors for cancer development. Dendritic cell EVs have MHC class II on their surface and can transfer MHC class II/antigen complexes to antigen presenting cells which in turn elicit T cell activation [49–54]. Plasmacytoid dendritic cells (pDCs) transfer antigens through EVs to conventional dendritic cells, enabling cross priming of CD8+ T cells [55]. EVs, as opposed to donor cells, are the major facilitators of MHC cross-dressing that promotes alloimmune responses to heart and islet transplantation [56]. Moreover, knockout of the EV secretion mediators Rab27a and Rab27b leads to chronic inflammation and inhibited responses to inflammatory signals [57], indicating that EVs may play a role in maintaining immunological homeostasis. Targeting of EV secretion by cancer cells has been proposed as a therapeutic target; however, broad targeting of EV secretion of all cells may have unwanted off-target effects that are tumor promoting. Consequently, a broader understanding of the functional contribution of EVs by non-cancer cells will provide critical insight into the feasibility of targeting EV secretion.

EVs serve a critical function in responding to infections and mediating cross-kingdom communication between the host organism and infectious agents. Transmissibility of a number of infectious agents, including HIV, noroviruses, rotaviruses, enteroviruses, malaria, prions, and anthrax, is impacted by EVs [58–63]. In the context of infection, IL-35 on Treg derived EVs promotes infectious tolerance by stimulating non-Tregs to produce IL-35 and by promoting B and T cell exhaustion [64]. Interactions between the tissue microbiome and immune cells mediated by EVs has been implicated in several inflammatory disorders [65–68]. The bidirectional cross talk mediated by EVs between host cells and the microbiome is likely important for tissue homeostasis and in mediating the immune response to inflammatory conditions, including cancer.

The role of EVs in inflammation, obesity, and cancer initiation

Chronic inflammatory disorders such as diabetes, pancreatitis, fibrosis, and non-alcoholic steatophepatitis are all risk factors for cancer development. Pancreatic islet cells release autoantigens in EVs in response to ER stress that stimulate T cell activation [69]. β cells secrete miRNAs in EVs in response to cytokines that can induce apoptosis in recipient

cells [70]. In addition, the proinflammatory β cell EV cargo can lead to dysfunction of recipient β cells and recruitment of macrophages and T cells, promoting disease progression [71]. Islet EVs increase the expression of cytokines secreted by Th1, Th2, and Th17 cells and increase the production of autoantibodies associated with type I diabetes [72]. Chronic inflammation and fibrosis can modulate the tissue microenvironment to promote cancer initiation. Pancreatitis lead to increased EVs in circulation and such EVs activated macrophages into a pro-inflammatory phenotype [73]. Moreover, plasma EVs from patients with severe pancreatitis elicited activation of NFκB signaling, expression of TNFα and IL1β, and generation of free radicals in macrophages [74]. *Helicobacter pylori*, the causative agent of gastritis, produce EVs that stimulate the secretion of TNFα, IL6, and IL1β by macrophages and IL8 by gastric epithelial cells to induce inflammation known to drive tumorigenesis [75].

Obesity is a risk factor for cancer, potentially through inflammation induction [76]. In early onset obesity, macrophage derived EVs containing miR-690 and hepatocyte derived EVs containing miR-3075 act to promote insulin sensitivity [77,78]. In contrast, in chronic obesity, EVs promote insulin resistance through proinflammatory signaling [77], suggesting that EV release is initially protective and is subjugated in chronic obesity to promote disease progression. High fat diet and high caloric intake drives the initiation of nonalcoholic steatohepatitis (NASH) in mice, which is characterized by excessive fat accumulation, fibrosis, and inflammation in the liver and is a risk factor for developing liver cancer [79]. In healthy livers, miR-690 is transferred from Kupffer cells to hepatocytes and stellate cells through EVs and acts to prevent the development of NASH and NASH is associated with loss of miR-690 in Kupffer cells [80]. NASH typically leads to lipotoxicity and ER stress that is mediated by inositol-requiring enzyme-1A (IRE1A) [81]. IRE1A stimulates the transcription of serine palmitoyltransferase genes to increase the release of hepatocyte derived EVs and drive inflammation [82]. Hepatocytes treated with the toxic lipid mediator lysophosphatidylcholine secrete EVs enriched with β1 integrin that increase proinflammatory monocyte adhesion to liver sinusoidal cells [83]. Thus, EVs have context-dependent roles in cancer initiation, with both restraining and promoting cancer initiation.

The early genetic drivers of cancer initiation can impact EV secretion, cargo packaging, and entry into recipient cells. Malignant cells typically have higher EV secretion compared to non-malignant cells, which is likely mediated by the mobilization of calcium from the ER [84]. Moreover, activation of p53 in response to stress is associated with increased EV secretion through TSAP6 [85]. The oncogenes AURKB, MYC, and HRAS[G12V] alter EV release, size, and their protein and miRNA composition [86]. Mutant RAS also induces the entry of EVs into cancer cells through macropinocytosis [87–89]. Cellular transformation with oncogenic HRAS induces the release of EVs containing oncogenic DNA [90]; however, the precise impact of EV associated DNA on recipient cells is not fully understood and whether such transfer occurs in vivo is not known. Mutant KRAS inhibits the accumulation of Ago2 in multivesicular endosomes and EVs, modifying the packaging of miRNA in EVs [91]. A number of oncogenic miRNA have been identified that have critical roles in tumor initiation and progression [92]. Breast cancer EVs are capable of processing precursor miRNAs into mature miRNAs, and transfer of EV associated miRNAs is sufficient to drive the transformation of nontumorigenic epithelial cells [93]. While such studies have implicated

EVs in processes that may increase the risk of developing cancer, currently evaluating the direct contribution of EVs to tumor initiation is difficult due to a lack of cell lines derived from precursor lesions and no specific EV markers of tumor initiating cells. Models that enable the study of such early lesions will provide clarify this point and provide potential early biomarkers of disease.

The functional contribution of EVs to cancer progression

A signaling network involving cancer cells and non-malignant cells, including epithelial cells, cancer associated fibroblasts (CAFs), endothelial cells, neurons, and immune cells, is critical for driving as well as restraining cancer progression. Transfer of EVs between cancer cells and stromal cells has been identified as a mechanism to reprogram the host tissue to alter tissue homeostasis and aid cancer progression (Supplementary Table 1–2). Pancreatic cancer cell EVs are enriched for biomolecules that elicit ER stress in non-tumorigenic recipient cells, potentially promoting their transformation [94]. PTEN is packaged in EVs and transferred between cells to inhibit Akt signaling and proliferation [95], suggesting a mechanism by which EVs from nonmalignant cells limit cancer cell proliferation. A dynamic transfer of EVs between cancer cells and other cells in the TME likely exists, and the balance of cancer cell EV secretion compared to TME cell EV secretion, as well as the cargo of such EVs, could ultimately determine cancer progression. Cancer cells generally have increased EV release compared to non-tumorigenic cells in the context of in vitro two-dimensional tissue culture plastic; however, this remains to be validated in vivo where the tissue microenvironment is more complex.

Cancer cell EVs can also transfer a number of immunomodulatory factors that impact antitumor immunity. Natural killer (NK) cell EVs carry cytotoxic proteins that elicit cancer cell killing which may act to limit cancer progression [96]. Multiple myeloma (MM) cells secrete EVs with the NKG2D ligands that initially activate NK cells; however, with prolonged exposure to MM EVs, NKG2D is downregulated leading to hindered NK function [97]. This suggests that the initial response to cancer cell EVs may be to induce the antitumor activity of immune cells, but such response can be ultimately subjugated by cancer cells to promote immune escape and disease progression. Cancer cell EVs have been implicated in promoting an immunosuppressive TME through suppression of T cells [98,99] and dendritic cells (DCs) [100,101], and promoting the pro-tumorigenic functions of macrophages [102] and myeloid derived suppressor cells (MDSCs) [103]. In contrast, subcapsular sinus CD169+ macrophages internalize cancer cell EVs, preventing their interaction with tumor promoting B cells [104]. This suggests that in some instances, EV entry may act as a functional sink preventing the delivery of EV cargo to other cell types. The fate of EV cargo in recipient cells and mechanisms controlling the targeting of EV cargo for degradation as opposed to retention are currently not completely understood.

The communication axis between cancer cells and CAFs mediated by EVs also impacts tumor growth and the immune microenvironment. Cancer cell EVs containing factors such as TGFβ, miR-125b, and mutant gain-of-function p53 are transferred to fibroblasts to induce CAF activation and promote cancer growth [105–108]. Activated NOTCH-MYC signaling in CAFs elicits secretion of unshielded RN7SL1 RNA in EVs that is transferred

to breast cancer cells, driving expression of the RNA pattern recognition receptor RIG-I and promoting tumor progression [109]. It is possible that other stromal cell types can contribute immunogenic RNA associated with EVs and that stromal ssDNA and dsDNA in EVs can elicit innate immune responses; however, this remains to be validated. miR-21, miR-378e, and miR-143 in CAF EVs promote the expression of EMT and cancer stem-like cell genes in breast cancer cells [110]. CD9 on CAF EVs is critical for entry into pancreatic cancer cells and pancreatic cancer progression [111]; however, pancreatic CAF EVs have also been reported to contain tumor suppressive miRNAs [112], potentially reflecting the functionally heterogeneous populations of CAFs that exist [113]. Currently, the contribution of EVs derived from distinct subsets of TME cells remains largely unknown. Experimental models that enable tracking and functionally interrogation of EVs secreted by TME cells will unravel their contribution to cancer progression.

EVs in mediating cancer metastasis

During metastatic progression, cancer cells disseminate from the primary tumor and colonize distant organs. Acquisition of phenotypes that promote escape from the primary tumor, extravasation at secondary sites, and subjugation of the metastatic stroma enable metastasis. In cancer cells that are local invading, EV secretion is increased at invadopodia and such secretion promotes adhesion assembly and is required for directional migration [114,115]. Both local and systemic exchange of mRNAs associated with cancer cell EVs occurs, potentially leading to transfer of metastatic behavior between cancer cells [116]. Further, the entry of EVs into recipient cells and their impact on cell proliferation is dependent on cancer cell metastatic state [117]. Live imaging of zebrafish embryos revealed that cancer cell EVs that are released into circulation enter endothelial cells and macrophages and cancer cell EVs can activate macrophages to facilitate metastatic outgrowth [118]. Intravital imaging of EV release and entry in recipient cells in larger scale mammals such as rodents has remained elusive and as a result, the fate of endogenously released EVs in cancer is largely unknown. Advances in imaging technologies may provide methodologies to track EV fate and unravel their functional impact.

At future metastatic sites, EVs can remodel the microenvironment to create a niche that is permissive for metastatic outgrowth (Figure 3, Supplemental Table 1–2). EVs can impact the vasculature and accumulation of bone marrow derived cells (BMDCs) to enhance metastatic colonization [119,120]. In addition to hematogenous spread, cancer cells also initiate metastasis through lymphatics. Melanoma EVs promote ECM deposition and angiogenesis to facilitate metastatic colonization of sentinel lymph nodes [121]. There is evidence of tissue-specific accumulation patterns of EVs that are reflective of future sites of metastasis, with EVs expressing $\alpha_6\beta_4$ and $\alpha_6\beta_1$ integrins associated with lung metastasis and $\alpha_v\beta_5$ integrin associated with liver metastasis [122]. While EVs accumulate in sites of metastasis, there is also EV accumulation in organs where metastasis typically does not occur, such as the pancreas, kidney, heart, bladder, and muscle [123]. In addition, mechanisms to limit the entry of EVs at metastatic sites have been identified [36,124] and bidirectional communication between cancer cells and the microenvironment is likely critical for metastatic progression. While the role of EVs in promoting metastatic dissemination is well-documented, mechanisms that limit the systemic transfer of EVs are not completely understood and

additional metastasis-independent functions of EVs in organs where metastasis does not occur are likely to be uncovered.

EVs as biomarkers of cancer and therapeutic response

EVs contain nucleic acids, proteins, metabolites, and lipids from the cell of origin and are present in circulation and other bodily fluids, and as a result have emerged as non-invasive biomarkers for disease and response to therapy (Figure 4). A 3 gene expression assay with urine EVs (ExoDx Prostate IntelliScore) can discriminate higher grade prostate cancers (Gleason score 7 or greater) from lower grade tumors and benign disease [125,126] and received a Breakthrough Device Designation by the FDA. Analysis of urine-derived EVs from prostate cancer patients revealed an enrichment of lncRNAs that are predicted to encode high-affinity neoantigens, which may be transferred to recipient cells and translated [127]. Moreover, metabolic differences were detected in urine-derived prostate cancer EVs compared to benign prostate hyperplasia EVs, indicating that urinary EVs can be used to non-invasively monitor the metabolic state of prostate tumors [128]. Urine EVs have emerged as a source of biomarkers for urological cancers; however, their utility for detection of other cancer types is less known. In addition, EVs derived from stool contain both human and bacterial ribosomal RNA [129], suggesting that stool EVs could be used to non-invasively monitor the evolution of gastrointestinal cancers and the microbiome simultaneously. Future studies evaluating stool EVs as a source of cancer and microbiome biomarkers are needed to determine their feasibility and accuracy for cancer detection and monitoring.

In addition to RNAs, DNA has been identified in EVs and its utility as a cancer biomarker explored. Common pancreatic cancer cell mutations, including $KRAS^{G12D}$ and $TRP53^{R273H}$, are detected in the DNA derived from EVs in circulation of pancreatic cancer cell patients [30,130–132]. Further, glypican 1 (GPC1) was identified as an early-stage marker of pancreatic cancer, and $KRAS$ mutations are detected in GPC1$^+$ EVs [133]. While DNA within the lumen of cancer cell EVs has low abundance [18], sequencing of such samples revealed higher coverage, indicating that DNA incorporated in EVs has improved utility compared to other cfDNA isolates.

Proteins present in EVs can enable cancer-specific EV capture and detection. Proteomic analysis of EVs derived from tissue explants, plasma, and bodily fluids identified CD9, HSPA8, ALIX, HSP90AB1, ACTB, MSN, and RAP1B as potential pan-EV markers and VCAN, TNC, and THBS2 as cancer-specific EV markers [134]. An advantage of EVs over other biomarkers used for cancer detection and monitoring, including soluble proteins, is that EVs contain multiple biomolecules that can be measured, potentially providing increased sensitivity and specificity. Sensors that simultaneously measure EV proteins and miRNAs as well as protein expression and activity have been developed, allowing for multiplexed analysis of EVs and potentially more accurate detection of cancer EVs [135,136]. Improved detection systems that are capable of multiparametric analysis, especially of individual EVs, that are capable of measuring EV heterogeneity are likely to emerge in the future.

EVs have also been evaluated for their utility in tracking responses to therapy. PD-L1 packaged in EVs inhibits T cell activation to promote immunosuppression [98,99] and

analysis of plasma EVs from melanoma patients revealed that increases in EV-PD-L1 are associated with disease progression and better predictive value compared to tumor biopsies [137]; however, the predictive power of EV based assays is currently limited by the availability of accurate biomarkers. The transcriptional profile of plasma EVs correlates with tumors in melanoma patients and can be used to predict response to immune checkpoint blockade. Deconvolution models were employed to predict the contribution of EVs derived from various melanoma tumor microenvironment cell sources to EVs in circulation [138]. Such analyses could be further expanded to profile EVs secreted by cells in the tumor microenvironment and understand their role and predictive power in cancer progression and response to therapy.

Therapeutic responses mediated by EVs

Cancer cells develop a variety of resistance mechanisms in response to therapy, including cell intrinsic and extrinsic mechanisms, and the transfer of miRNAs and lncRNA through EVs can confer chemoresistance to other cancer cells (Supplemental Table 3–4). EV-mediated therapy resistance can potentially act through distinct but not mutually exclusive mechanisms, including transfer of proteins and miRNA that promote therapy resistance [139–141], transfer of drug transporters [142], acting as decoys for antibody-based therapeutics [99], and by preventing antibodies from accessing their ligand target [143]. EV secretion is also postulated to be a mechanism of removal of unwanted cellular materials, suggesting that drugs may be packaged into EVs, limiting their functional impact on cancer cells. The lncRNA lncARSR (lncRNA Activated in RCC with Sunitinib Resistance) is incorporated in sunitinib resistant RCC EVs and can transmit resistance by competitively binding miR-34/miR-449 to induce AXL and c-MET expression [144]. EVs from GBM cells transfer spliceosomal proteins and snRNA to recipient cells to impact transcription in recipient cells, promoting therapy resistance [145]. Cargo packaged in EVs from stromal cells, including CAFs, endothelial cells, and immune cells, have been implicated in therapy resistance. Noncoding RNA and transposable elements in CAF EVs are transferred to breast cancer cells, where they induce pattern recognition and antiviral signaling and activate NOTCH3 to promote therapy resistance [146]. Moreover, CAF EVs contain mitochondrial DNA that is transferred to cancer cells to induce oxidative phosphorylation, an escape from dormancy, and resistance to hormone therapy in breast cancer [147]. The relative contribution of EVs and their cargo acting as decoys to influence therapeutic responses in comparison to direct transfer of EV cargo to therapy resistance is not currently known and warrants future investigation.

An effective therapy response elicits lasting antitumor immunity and crosstalk between cell compartments in the tumor microenvironment is critical for establishing such memory responses. Vitamin E treatment enhanced DC function through inhibition of SHP1 and increased antigen presentation by DCs and DC derived EVs to elicit antitumor immunity [148], suggesting that EV release can be modulated to promote effective therapy responses. While CAR-T cells have demonstrated effective control of a number of hematopoietic cancers, solid tumors are largely non-responsive to CAR-T cell therapy, in part due to microenvironment factors [149]. CAR-T cells engineered to express RN7SL1 transfer EVs containing RN7SL1 RNA to myeloid cells to inhibit the MDSC phenotype and to DCs to

promote costimulation, promoting CD8 T cell mediated clearance of solid tumors [150]. This suggests that the efficacy of adoptive cell transfer (ACT) therapies is in part dependent on EV transfer, creating a potential opportunity to improve ACT through modulation of EVs.

The development and clinical testing of EV-based cancer therapeutics

The membrane of EVs can protect intraluminal cargo and the surface proteins in unmodified EVs act to prolong circulation times and accumulation in specific organs, especially tumors and the liver and spleen [88,151]. Moreover, EVs are large enough to presumably avoid renal clearance [152]. As a result, a number of small molecule drugs have been incorporated into EVs for delivery of therapeutic payload to tumors while limiting off-target effects (Figure 5). The chemotherapeutics paclitaxel, doxorubicin, and gemcitabine have been packaged in EVs and demonstrated effective suppression of tumor growth [153–158]. In addition, EV based delivery vehicles with siRNAs targeting $KRAS^{G12D}$, MYC, $S100A4$, and $PAK4$ have been employed [88,151,159–161]. EVs engineered to incorporate antisense oligonucleotides [162], CRISPR/Cas9 [163–165], and miRNA [166,167] have also elicited effective tumor growth control. In order to further improve loading of cargo into EVs while maintaining the biocompatible properties of EVs, hybrid vesicles incorporating both EVs and synthetic materials have been developed [155,168–172]. In depth analysis of the immune responses and off-target effects of such strategies will provide critical insight into the clinical feasibility of hybrid and other nanovesicles. In addition, while these engineering strategies may improve cargo loading or targeting to specific tissues, therapeutics with increasing complexity can also create additional regulatory hurdles for clinical translation. As a result, the tradeoffs between such hurdles and engineering benefits need to be weighed for the successful implementation of EV based therapeutics.

EVs can modulate immune cell function and consequently exploiting such interactions therapeutically has been proposed for the control of cancer progression. STING agonists have demonstrated the ability to stimulate anti-tumor immune responses; however, clinical translation of STING agonists have been limited by bioavailability issues and off-target toxicity. Incorporation of small molecule STING agonists in EVs effectively activates antigen presenting cells and anti-tumor immunity with lack of off-target effects [173,174]. Stimulation of the RIG-I pathway leads to type I interferon secretion and an anti-tumor response, but RIG-I agonists have similar limitations to STING agonists in vivo. Incorporation of RIG-I agonists in red blood cell EVs stimulates immune responses and suppresses tumor growth [175], further demonstrating the potential for incorporating immune modulatory molecules in EVs.

A number of cell-based immunotherapies have been developed and demonstrate effective control of tumor growth, including DC vaccines and CAR T cells. Despite their efficacy, cell-based therapies have several limitations, such as development of immunosuppressive mechanisms, off-target toxicities, and the need for autologous cells. EVs have emerged as cell-free immunotherapies that can circumvent many of the issues associated with cell-based therapies. DC derived EVs contain functional MHC class I/peptide complexes which can prime T cells to elicit anti-tumor responses [176]. As a result, DC EVs have been proposed as cell-free vaccines for cancer. Small EVs or exosomes from ovalbumin-pulsed dendritic

cells induce antigen-specific CD8+ T cells, whereas large EVs or microvesicles do not [177], indicating that small EVs are more effective at eliciting antigen-specific immune responses. EVs derived from DCs pulsed with a cancer-specific aberrant transcription induced chimeric RNA, potentially providing an EV based vaccination strategy for cancers that lack a known mutational antigen [178]. Such strategies could be expanded to readily modified EVs to incorporate RNA molecules and/or proteins to vaccinate against various cancer mutations. In addition to DC EVs based vaccination strategies, EVs from other immune cell types have been investigated as cancer therapeutics. EVs from CAR-T cells (CAR-EVs) express CAR on their surface and are capable of inducing cytotoxicity and tumor growth inhibition [179], suggesting that CAR-EVs can act as cell-free immunotherapies. Although CAR-EVs have therapeutic promise, whether autologous EVs are required to prevent graft-versus-host responses is not currently known. Allogenic NK CAR cell therapies have been employed to circumvent this issue and EVs from NK cells contain cytotoxic proteins and demonstrate cancer cell killing capacity [96], suggesting that EVs from NK CAR cells may be an effective immunotherapy. Together, these studies provide strategies for controlling tumor progression via off-the-shelf EV based immunotherapies.

The translation of EV based therapeutic vehicles requires large scale, GMP production. Bioreactors enable large scale culture of cells under defined conditions for EV isolation. Several strategies have been employed to generate clinical-grade EVs based on differential ultracentrifugation [151], density gradient ultracentrifugation [180], and tangential flow filtration (TFF) [181]. TFF and size exclusion chromatography (SEC) allow for isolation of EVs from larger volumes of cell culture media, potentially more readily enabling broad clinical application of EV based therapeutics. EV based therapeutics face many of the same challenges associated with the clinical translation of cell-based therapeutics, including characterization of the cellular source of EVs, EV isolation and storage, and quality control and standardization. Phase I trials of DC EV cancer vaccines (Dex) concluded with lack of toxicity and an objective response in one patient [182]. In non-small cell lung cancer, a phase II clinical trial of Dex concluded that Dex is well tolerated but did not meet its primary endpoint of 50% of patients with progression-free survival at 4 months post-chemotherapy [183]. More recently, several EV based therapeutics for cancer initiated clinical testing. Two phase I trials evaluating EVs incorporating STING agonists (exoSTING, NCT04592484) and IL-12 (exoIL-12, NCT05156229) completed recently. exoIL-12 demonstrated a manageable safety profile in healthy subjects and cutaneous T cell lymphoma patients and the recommended phase 2 dose was identified (NCT05156229). The safety and tolerability of EVs with STAT6 ASOs (exoASO-STAT6) are currently being evaluated in patients with hepatocellular carcinoma and gastric and colorectal cancer metastasis to the liver (NCT05375604). In addition, the safety and efficacy of EVs with KRASG12D targeting siRNA (iExosomes) are being determined in ongoing phase I trials in metastatic pancreatic cancer patients (NCT03608631). Thus far, EV cancer therapeutics appear to be safe and well tolerated, and ongoing trials will provide additional insight into the efficacy of different EV therapeutic modalities.

Perspectives and future directions

Significant advances have been made in recent years that have enabled unprecedented insight into EV biology and function in cancer progression, response to therapy, and metastasis. Our understanding of the function of EVs is predominantly in perturbed systems, i.e. disease states, and the role of EVs in normal physiology and homeostatic tissue function remains elusive. Moreover, precancerous cell types are typically difficult to expand ex vivo and maintain their phenotypes, precluding EV isolation and analysis to evaluate EV contribution to cancer initiation. Similar challenges exist with certain cells in the tumor microenvironment, e.g., lymphatic endothelial cells, neurons, and subsets of immune cells and CAFs. Markers enriched in circulating EVs from early-stage cancer patients and normal individuals have been identified; however, the precise cellular origin of such EVs are not known. Consequently, models that enable the tracking of EVs released by distinct cell populations in vivo will help to clarify these points. In addition, several mediators of EV biogenesis have been identified in vitro, but whether these functions are conserved in vivo and restricted to EV secretion are unknown. The identification of EV-restricted mediators of secretion will more readily enable the functional dissection of the contribution of EVs to cancer progression.

Single EV analysis techniques revealed that individual EVs display heterogeneity in their size [184] and marker expression [185–187]; however, the majority of studies focused on understanding the role in EVs in cancer are based on EVs isolated using crude methods that presumably capture a mixture of heterogenous EVs. EVs have differential impacts on metastatic outgrowth and biodistribution based on bulk measured surface markers and size [24,122] and CD63^{+} EVs contain both common and non-overlapping protein cargo compared to CD9^{+} EVs [5]. Consequently, distinct functional subsets of EVs likely exist. EV technologies have expanded rapidly in recent years to include single EV analysis and sorting as well as methods to isolate EVs based on their size and charge, which will enable the profiling of EV subsets and evaluating their functional role. Moreover, the development of novel techniques to measure nucleic acids in single EVs will further elucidate EV heterogeneity.

Clinical trials of EV based therapeutics thus far have not revealed significant toxicities and unmodified EVs from certain nonmalignant cell types are immunologically inert and can be used as allogenic therapeutics [151,188]. Modification of EV cargo to express CD3 antibodies reprograms EVs to activate T cells [189], suggesting that EVs could be further engineered to generate off-the-shelf allogenic therapeutics with defined immune-targeting and/or immunomodulatory properties. as vaccines [190] a strategy which could be further exploited to generate EV vaccines with cancer antigens. Such strategy could be used for personalized medicine to target patient-specific mutations as well as more broadly occurring mutations such as KRAS[G12D].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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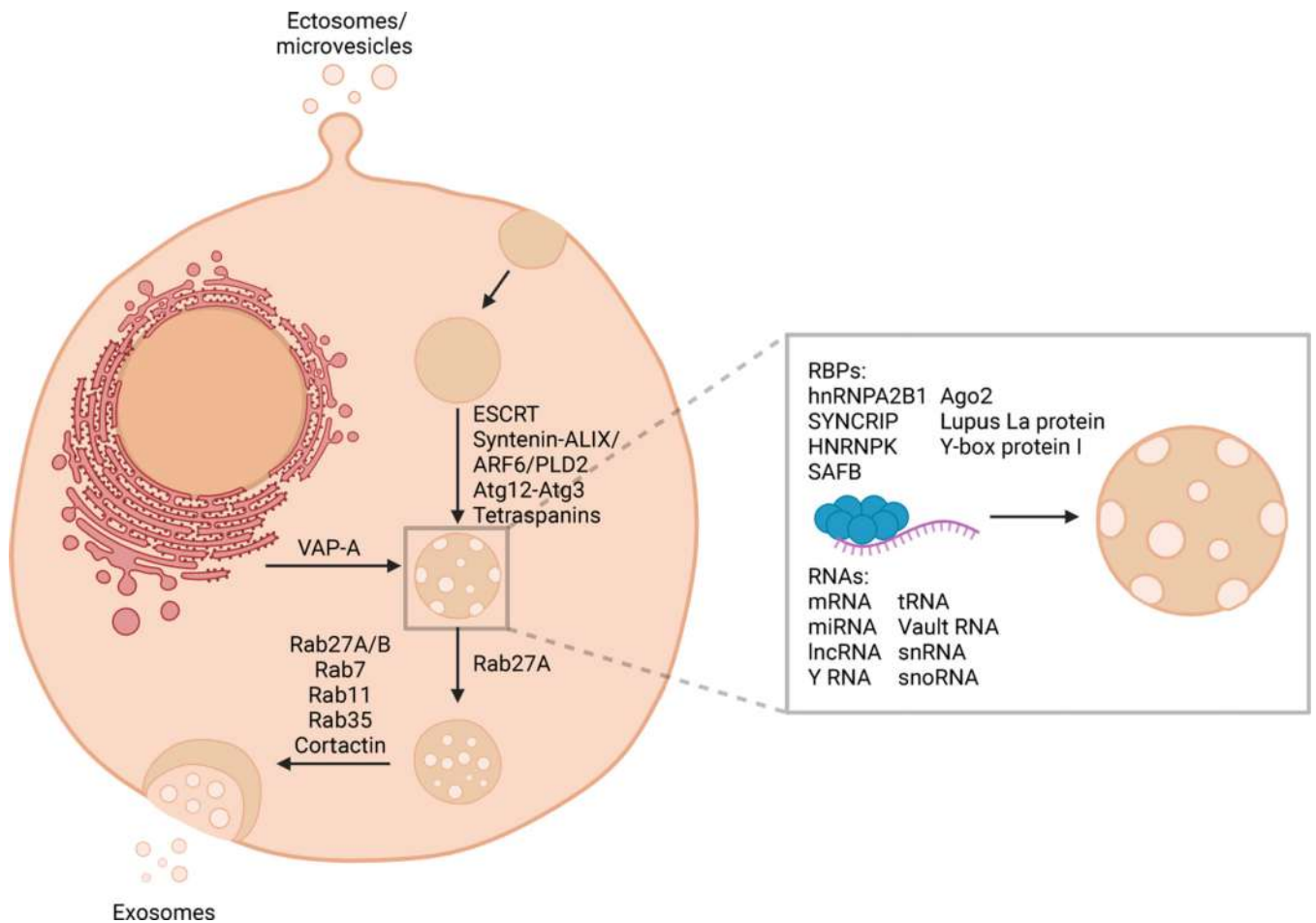
Figure 1. Extracellular vesicles include exosomes and ectosomes or microvesicles.
EVs present with a phospholipid bilayer membrane oriented similarly to that of the plasma membrane of the cell they are generated from. Exosomes are generated via the endosomal pathway, and result via the sequential invagination of the plasma membrane forming multivesicular bodies before they are released extracellularly. Ectosomes/microvesicles are generated via the outward budding of the plasma membrane. The mediators of different stages of multivesicular body formation, maturation, and release and RNA packaging into EVs are labeled. RBPs, RNA binding proteins. Created with BioRender.com.
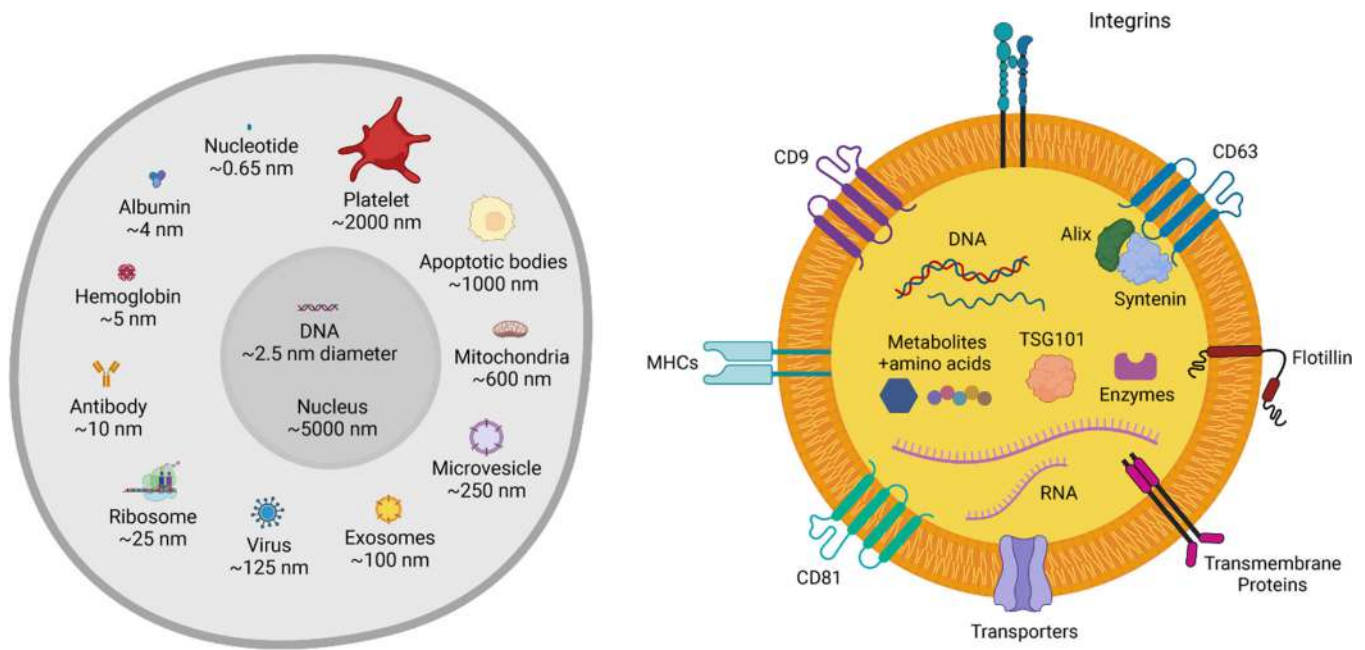
Figure 2. Relative size and cargo of EVs.

(**A**) Average size of exosomes and microvesicles with respect to cellular components, including abundant proteins (albumin, hemoglobin, antibody), organelles (ribosomes, mitochondria), nucleotides and DNA, virus, as well as cellular byproducts: apoptotic bodies and platelets. (**B**) Composite cargo of exosomes, including surface receptors (protein, glycoprotein, glycans, ion channel receptors, G-protein coupled receptors, enzyme-linked receptors, integrins, etc.), transmembrane proteins (FasL, PD-L1, etc.), intracellular proteins, metabolites, lipids, and nucleic acids (RNA: mRNA, pre-/miRNA, piRNA, tRNA, snRNA, snoRNA, Y-RNA, circRNA; DNA: dsDNA, ssDNA, mtDNA, foreign DNA; cAMP). Created with BioRender.com.
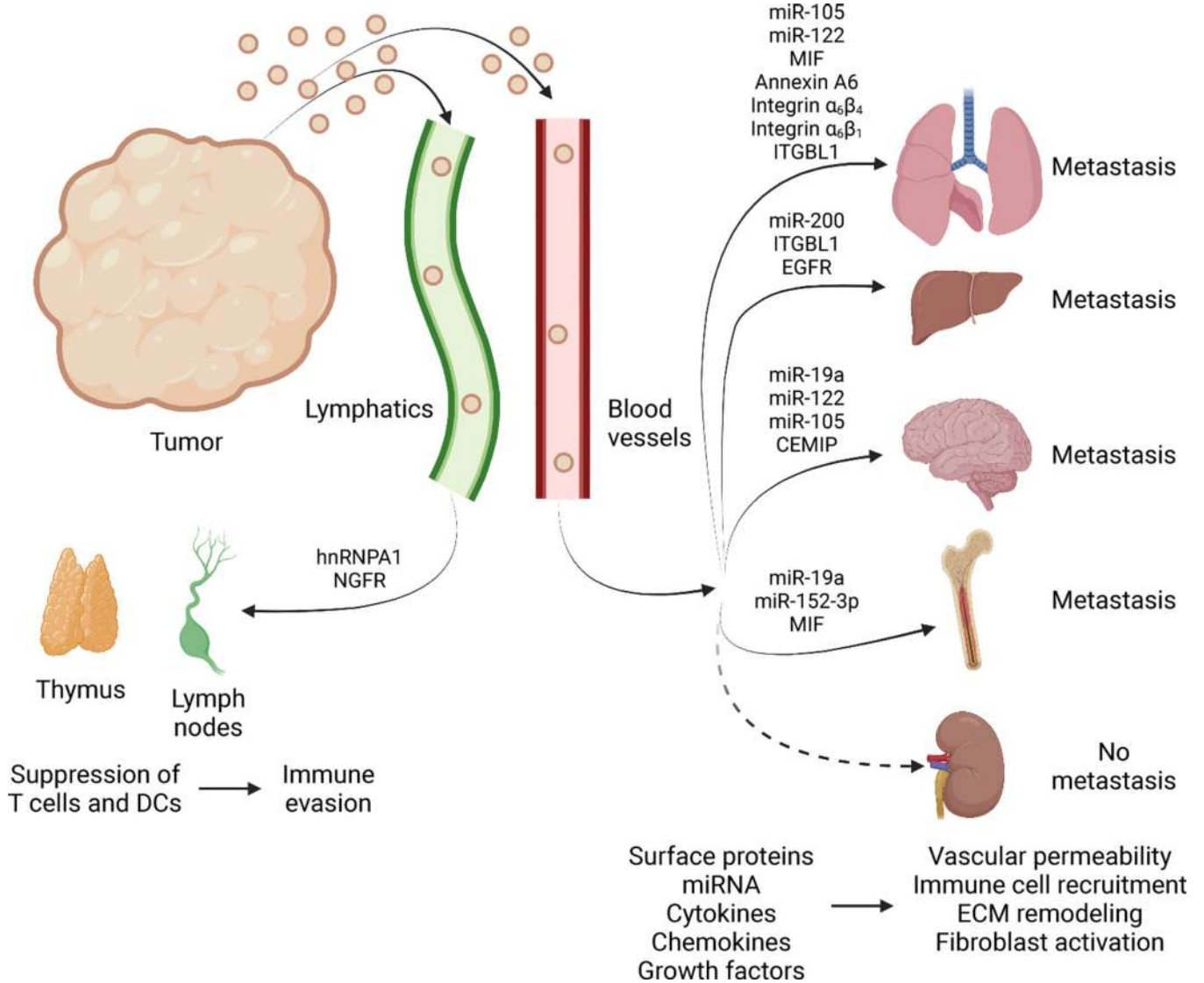
Figure 3. Extracellular vesicles in metastatic disease.
Tumors release EVs, from both cancer cells and host cells of the tumor microenvironment (TME) into systemic circulation using both lymphatic and blood vessels. EVs interact with lymphoid organs including thymus and lymph nodes, with impact on T cell activation, DCs, and possibly aiding immune evasion. EVs also influence metastasis to lungs, liver, brain, and bone and possibly other non-metastatic sites by modifying vascular permeability and impacting immune cell recruitment, extracellular matrix (ECM) remodeling, and fibroblast activation. EVs exert their function by altering recipient cells via delivery of RNA, cytokines, chemokines, or growth factors, or surface protein signaling. Created with BioRender.com.
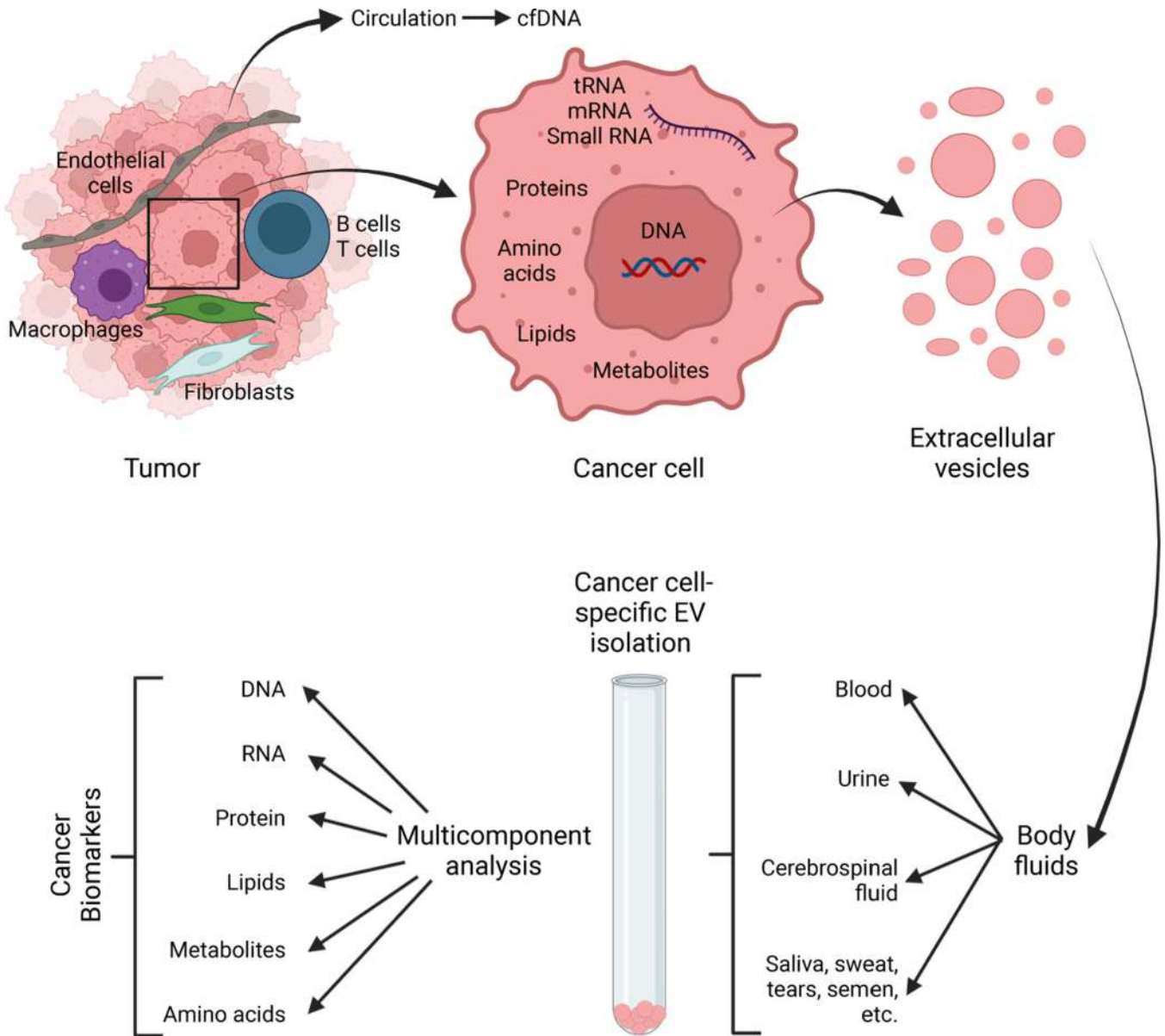
Figure 4. EVs as cancer biomarkers.

Cancer cells shed EVs with a characteristic cargo representing a range of cancer cell components, including nucleic acids, proteins, lipids, metabolites etc. EVs are found in all body fluids, including blood, urine, cerebrospinal fluids, saliva, sweat, tears, semen etc..) and may be enriched with various isolation protocols. EVs lend themselves to a multicomponent analysis reflecting a collection of cancer cells byproducts for biomarkers study, which likely offer a more comprehensive readout when compared to ctDNA analysis alone. Created with BioRender.com.
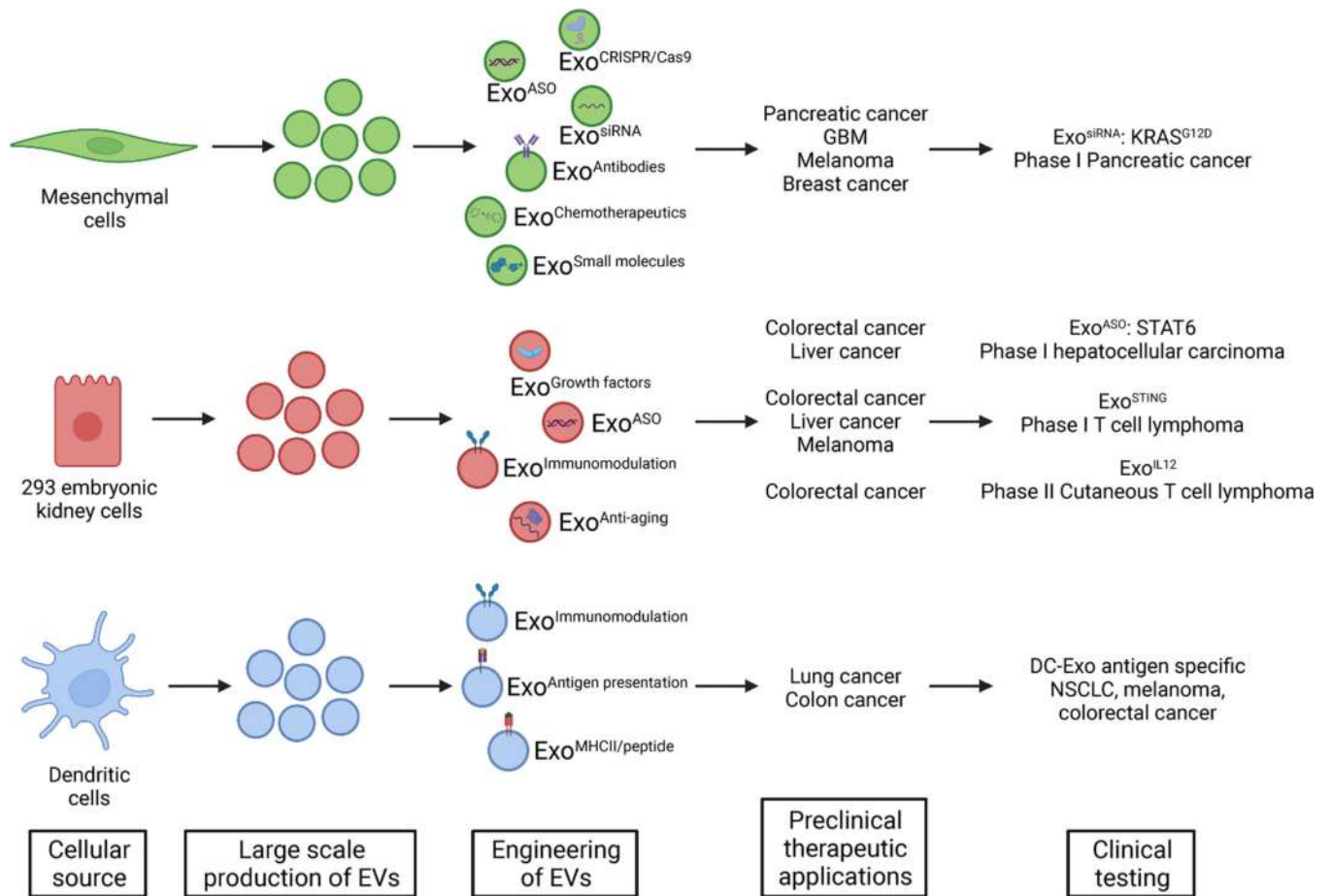
Figure 5. EVs as anti-cancer therapeutic agents.

Distinct cellular sources have been used to generate EVs in large scale for clinical trials. EVs engineering include the incorporation of a cargo (e.g. ASO, siRNA, chemotherapeutics etc), enriching for exosomes with unique surface protein presentation (e.g. antigen, immune modifying receptor). Preclinical studies in various tumor models and tumor types informed ongoing clinical trial design. EVs offer a novel therapeutic platform for cancer treatment, from personalized medicine to immunotherapy and targeted therapy with novel safety and efficacy profiles. Created with BioRender.com.