Chapter 8
EV, Microvesicles/MicroRNAs and Stem Cells in Cancer

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Abstract The role of extracellular vesicles (EV) in carcinogenesis has become the focus of much research. These microscopic messengers have been found to regulate immune system function, particularly in tumorigenesis, as well as conditioning future metastatic sites for the attachment and growth of tumor tissue. Through an interaction with a range of host tissues, EVs are able to generate a pro-tumor environment that is essential for tumorigenesis. These small nanovesicles are an ideal candidate for a non-invasive indicator of pathogenesis and/or disease progression as they can display individualized nucleic acid, protein, and lipid expression profiles that are often reflective of disease state, and can be easily detected in bodily fluids, even after extended cryo-storage. Furthermore, the ability of EVs to securely transport signaling molecules and localize to distant tissues suggests these particles may greatly improve the delivery of therapeutic treatments, particularly in cancer. In this chapter, we discuss the role of EV in the identification of new diagnostic and prognostic cancer biomarkers, as well as the development of novel EV-based cancer therapies.

Keywords Extracellular vesicles · Non-coding RNA · Cancer · Mesenchymal stem cell · Exosomes · miRNA

8.1 EV as Novel Cancer Biomarkers

The need for novel cancer biomarkers is fundamental in improving patient outcomes. This search has resulted in the emergence of EV as new predictive, diagnostic, and prognostic factors in cancer. EV can be obtained from virtually any body fluid or tissue, by safe and minimally invasive or non-invasive methods.

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Additionally, the intrinsic nature of EV protects the internalized (and external, to a degree) contents from host and environmental degradation, allowing easier EV isolation and storage. As EV are often released in higher concentration from tumor tissue, and the expression profile often mimics/reflects host cell expression profiles, they can be used as a liquid biopsy of the cancer tissue, even tissue that is unreachable via conventional methods [1–4]. EV may also be used as a future indicator of disease in healthy populations, leading to improved health planning and patient outcomes. This aids in determining the most effective treatment options, resulting in decreased economic burden and fewer unwanted side effects in patients.

Two major challenges exist in the development of EV diagnostics and prognostics in cancer. The first challenge is our limited understanding of the spectrum of signaling options that are available due to the complexity of EV surface expression. The sometimes-low concentrations of certain EVs, as well as the diversity and heterogeneity of EV type and expression profile also hamper development [5–7]. This will be improved with biobanking of both healthy and diseased tissue for adequate comparative analyses [7]. This problem is common in emerging diagnostics/prognostics and requires substantial resources and investment to generate a reliable and affordable repository. The second challenge is the development of economical methods of isolating and analyzing EV from samples. Though the liquid biopsy is a safe and effective method, high-sensitivity methods of isolating and characterizing EV are only beginning to be established [7, 8].

EV contain a varied assortment of factors, that present significant diagnostic and prognostic potential in cancer treatment. For these purposes, EV are most often obtained from patient serum, though plasma and urine are also easily utilized [9, 10]. Factors isolated from EV not only discern healthy from diseased patients but can also be effective in staging disease. Many studies have identified EV nucleic acid, particularly miRNA, as an effective cancer biomarker [11–17]. These studies identified many indicative miRNA species in a vast array of cancers, often using quantitative PCR and/or sequencing for RNA detection [18, 19]. Undoubtedly many studies utilizing serum miRNA as diagnostic and prognostic disease markers have accidentally harvested exosomal miRNA. In fact, exosomal miRNA may represent a significant fraction of commonly isolated miRNA in some studies. Other nucleic acids that have been identified as demonstrating biomarker potential are mRNA, DNA (containing oncogenic mutations), short non-coding RNA, and circular RNA [20–25]. Much like EV miRNA, many studies have utilized mass spectrometry techniques to identify an array of proteins that are highly indicative of disease state [10, 26–28]. Protein markers have thus far demonstrated significant potential, with a recent study identifying a marker that displayed unprecedented accuracy in diagnosing and staging disease state in pancreatic cancer patients [29]. Analysis of the lipid composition of EV has shown lipid expression profiles may also be a potential cancer biomarker [30].

8.2 EV Biomarker Technology in Cancer

The future of EV as diagnostic and prognostic markers in cancer relies on the development of systems that rapidly capture and identify markers of disease. Common methods for isolating EV for biomarker analyses include standard isolation techniques based on filtration combined with ultracentrifugation, and immunoaffinity capture methods [6, 7]. Though effective, the cost of these technologies is currently prohibitive for large scale implementation [31]. Thus, new technologies are being developed to utilize the vast content of EV for therapeutic purposes. Recent developments in the modification of existing technologies used in liquid biopsy analysis have already provided new diagnostic methods [8, 31]. These include several effective immunoaffinity capture methods, including the *ExoChip*, *ExoScreen* and *ExoSearch* technologies, that allow rapid identification of specific EV markers associated with oncogenesis [32–34]. Fortunately, EV factors can be identified using a range of methods including PCR, mass spectrometry, nuclear magnetic resonance, and immunofluorescence [26, 35–41]. Two diagnostic EV technologies are currently available that identify RNA signatures in the urine of prostate cancer patients and the serum of lung cancer patients (www.exosomedx.com) [42, 43]. These markers help diagnose disease and determine treatment options. Although only two methods are currently available, many clinical trials utilizing EV-based technologies in cancer diagnostics are under investigation.

8.3 Novel Role of EV in Cancer Therapy

The burgeoning area of EV function in cellular communication derives from their ability to protect and transport a range of cargoes to a wide array of tissues [3, 19, 44–48]. This ability is being utilized in the development of novel therapies in the treatment of many diseases, particularly cancer [8, 49–51]. Most EV-based therapies utilized natively-derived (obtained from patients) or semi-synthetic/bioengineered EV (mimetics) that deliver compounds which either activate/enhance antitumoral immune responses (cancer vaccines) or deliver antiproliferative agents directly to the tumor tissue (therapy delivery) [52–69]. Apart from the aforementioned vaccination and therapy delivery, the removal of EV or inhibition of EV production to reduce cancer growth and/or pre-metastatic niche formation is also evaluated [70–74]. This has been investigated via the reduction of Rab27a protein expression, as well as the removal of circulating EV via filtration or immunoaffinity capture [6, 31, 75–78].

EV make excellent delivery vehicles due to their bioavailability and lack of unwanted immunogenicity. When compared with the delivery of soluble factors alone, EV-internalized or associated factors often display increased efficacy with minimal off-target/side effects [56, 79–81]. The complexity and hence similarity of

exosomal surface expression to host cells both increases the effectiveness of EV as delivery systems, as opposed to synthetic vehicles, and reduces unwanted immune responses due to their syngeneic nature [8, 80, 82]. This can result in increased uptake of exosomal contents by host cells compared to synthetic particles, such as liposomes [49, 50]. This is advantageous in the delivery of certain compounds, such as chemotherapeutics, where tumor uptake is enhanced (increased tumor cytotoxicity) while unwanted drug deposition is reduced (reduced side effects). This complexity also permits the encapsulation of multiple compounds that could target several cell types or targets.

However, there are also disadvantages to using biological EVs as therapeutic vehicles [8, 51]. Sometimes generalized increased uptake is not required, but more limited and specific uptake in certain sites or tissues. Although synthetic EV can have unwanted toxicity and immunogenicity, enhanced immunogenicity may be required to maximize antitumor effects. These issues require a modified delivery system that does not necessarily prevent uptake of the nanovesicle, but prevents content release unless the desired inter/intracellular conditions are met. With current technology, synthetic particles have been advantageous in this respect, as the regulation of surface expression is far easier, and the particle structure can be easily modified to prevent release at unwanted sites, such as low or neutral pH [83–86]. Thus, the two main advantages of synthetic and semi-synthetic EV delivery systems are that the manufacturing process limits unwanted variability/heterogeneity (an issue when utilizing current biological systems for EV generation), and that synthetic EV can be generated on large scale, suitable for drug delivery or vaccination. Future therapies will most likely rely on a combination of these methods, as well as the generation of EV mimetics, a type of EV of biological origin, generated via non-biological mechanisms [67, 68, 87, 88].

8.4 Generation and Modulation of EV for Cancer Therapy

As of 2016, there were no commercial EV-based therapies available for the treatment of cancer. Although synthetic nanovesicle delivery systems have been established in the treatment of array of diseases, the potential of EV to deliver therapeutic compounds is beginning to be elicited [8, 51]. The generation of EV to be used in cancer treatment relies, fundamentally, on two methods; the isolation of EV from the patient, tissue, or cell culture, followed by modification (drug, protein, nucleic acid, lipid) and reintroduction to the patient as treatment; or the large-scale isolation/fabrication of EV from cell culture, bioreactor or animal body fluid, again, followed by modification and introduction to the patient. *Ex vivo* modification of EV is often required to regulate antigen presentation or surface expression in order to modulate immunostimulatory potential and enhance selective uptake and delivery of EV contents [80, 85, 89, 90]. These contents can be internalized utilizing a range of methods. The cells used to generate the EV can be treated with factors that

regulate EV expression of protein and nucleic acid, and to produce exosomes that contain said factor [60, 85]. EV themselves can also be treated to incorporate specific contents. Simple incubation can facilitate uptake of certain compounds, while more complex methods, such as electroporation or enzymatic poration can also be used [49, 51, 62].

Though EV can be isolated from nearly all cell types and bodily fluids, exosome production for cancer therapy is limited. This includes primarily dendritic cells, cancer cells, and stem cells, each having distinct advantages and disadvantages. The first study to demonstrate the effectiveness of EVs as a mechanism for delivery showed that EV could deliver siRNA while effectively crossing the blood blain barrier [19, 57]. Though not a cancer treatment, the use of the host's EV for therapy propogated widespread interest in this method. In this study, dendritic cells were harvested and modified before reintroduction into the host, but these are not the only cell types that can be used in the production of therapeutic EV [19]. Regardless of the method utilized, substantial data indicates the necessity for diligent selection of the cell type to be used due to unwanted side-effects. These effects are intrinsic due to the heterogeneity in surface expression of EV.

Besides the significant changes in yield between and within these methods, the most important consideration is the surface expressed factors that dictate uptake, as complex EV expression profiles can obscure other functions [91]. The use of EVs as therapy requires the utmost stringency in the selection, isolation, and preservation to ensure patient safety. Exosomes derived from cancer cells tend to express higher levels (sometimes only) of MHC class I and a diverse array of growth factors, while EV from dendritic cells tends to express higher levels of MHC class II and lower amounts of growth mediators [8, 81, 92–97]. EV from mesenchymal stem cells (MSC) have been shown to be anti-inflammatory but can both enhance and inhibit tumor growth in different contexts [98]. Depending on whether the chosen method is to engage the immune system or directly kill tumor tissue, certain complications are inherent to EV-producing cell types and may have both positive and negative effects for the development of novel treatments. For example; aiming to generate an immune response that engages and destroys tumor tissue may have indirect proliferative effects on tumor tissue, while directly targeting tissue with EV cytotoxic drugs may compromise anti-tumor immune responses. Thus, modification of surface expressed factors is often required to elicit effectiveness, by improving immunogenicity or cytotoxicity.

Of the cell types discussed, MSC have shown the most potential, due to their low immunogenicity and ability to generate substantial quantities of EV [99, 100]. They are also relatively easy to obtain from patients allowing for personalized treatment. Recently, the use of bioreactors to culture adipose-derived MSC was shown to increase EV yield approximately 100-fold compared to conventional culturing methods [101]. Other methods for the large-scale purification of EV include harvesting from bovine milk, or the generation of EV mimetics, generated via serial extrusion [67, 68, 87, 88, 102]. This process generates nanovesicles of identical biological composition to EV, opening their potential for use in therapy.

8.5 Therapeutic Contents of EV in Cancer Therapy

Therapeutic contents of EV utilized in the treatment of cancer consist primarily of RNA or chemotherapeutics. Several studies have investigated the delivery of compounds via modified EV derived primarily from MSC. MSC-derived EV containing miRNA and anti-miRNA could increase sensitivity or re-sensitize tumor tissue to chemotherapeutics, and inhibit tumor growth [53, 64–66] . The efficacy of these methods can be improved my modifying the expression profile of the EV, resulting in greater uptake by target cells. The use of therapeutic siRNA is also being investigated, where preliminary studies have shown significant increases in mRNA depletion, leading to substantial decreases in cancer cell proliferation and viability [54, 56, 103]. EV, particularly from MSC, have also been used to enhance the effect of chemotherapeutics [58, 59, 61]. MSC treated with chemotherapeutics release large quantities of drug-containing EV. These EV can be more effectively used to deliver compounds to target cells [60]. Off-target effects can be further minimized by delivering modified EV that contain enzymes which activate prodrugs in tumor tissue [54]. Prodrug accumulation in other tissues is insignificant as the negligible levels of EV uptake by non-cancerous cells minimize drug activation. Currently, only two trials have investigated EV as method for drug delivery in cancer treatment, both utilizing plant-derived EV to either enhance the delivery of chemotherapeutics to tumor tissue (NCT01294072) or minimize side-effects of standard therapy (NCT01668849).

EV can also be utilized to deliver cargo that activates or enhances anti-tumor immune responses, producing a retroactive cancer vaccine [80, 95, 97, 104–106]. EV from tumor cells, and particularly dendritic cells, can contain be induced/modified to express/contain increased levels of MHC complexes for antigen presentation, as well as immunostimulatory components, such as heat shock proteins, interferon, and granulocyte macrophage colony stimulating factor [8, 81, 92–97]. These EV serve to enhance cytotoxic T-cell and Natural Killer cell responses against tumor tissue. Thus far, trials have investigated EV as an anti-cancer vaccine in lung (NCT01159288) and colorectal cancer, as well as malignant glioma (NCT01550523, NCT02507583). Studies investigating malignant glioma utilized a novel method for EV delivery. Rather than systemic delivery of EV, modified glioma cells captured within diffusion chambers were surgically inserted in the patient. As the glioma cells undergo apoptosis due to prior *ex vivo* modification, they release a range of vesicles, in particular EV, that serve to stimulate glioma-specific anti-tumor immune responses [107]. Although showing great promise, EV-based therapies for cancer have yet to make it to market.

8.6 Summary

EV are intriguing and present a new paradigm in our understanding of the dynamics of cancer pathology and treatment. Though the function of exocytosis in oncogenesis is not fully understood, many studies have demonstrated the capabilities of EV

in many aspects of cancer diagnostics and treatment. Though EV-based cancer treatments are still in clinical trials, EV-based biomarkers have recently become available for cancer diagnosis. With an increased understanding of the complex signaling potential of EV, combined with rapid and sensitive analysis methods, these nano-sized particles will undoubtedly provide a range of new options in cancer treatment.

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