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ADVANCED REVIEW

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Progress in extracellular vesicle biology and their application in cancer medicine

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Abstract

Under the broader category of extracellular vesicles (EVs), exosomes are now well recognized for their contribution and potential for biomedical research. During the last ten years, numerous technologies for purification and characterization of EVs have been developed. This enhanced knowledge has resulted in the development of novel applications of EVs. This review is an attempt to capture the exponential growth observed in EV science in the last decade and discuss the future potential to improve our understanding of EVs, develop technologies to overcome current limitations, and advance their utility for human benefit, especially in cancer medicine.

This article is categorized under:
Therapeutic Approaches and Drug Discovery > Emerging Technologies
Therapeutic Approaches and Drug Discovery > Nanomedicine for Oncologic Disease

KEYWORDS

biogenesis, cancer, exosomes, extracellular vesicles, microvesicles

1 | INTRODUCTION

The existence of submicron extracellular vesicles (EVs) has been known for more than 70 years (Chargaff & West, 1946). At the time of discovery, EVs were thought to be involved in different physiological activities, but not in cellular communication or other important activities (Anderson, 1969; Benz & Moses, 1974; Wolf, 1967; Figure 1). Later studies reported the role of EVs in cell signaling; however, much of the information regarding their structure, biogenesis, and function remained elusive until the 1980s, when two articles described the biogenesis of EVs in rat reticulocytes when multivesicular endosomes matured to late endosomes and were released from the cells (Harding, Heuser, & Stahl, 1984; Pan & Johnstone, 1983). In 1987, Johnstone termed these EVs "exosomes" (Johnstone, Adam, Hammonds, Orro, & Turbide, 1987). The precise biogenesis pathway and detailed structure of EVs/exosomes remained indeterminate until the late 1990s. The presence of EVs in the cell system was well accepted by cellular biologists, who considered EVs to be involved in removal of waste and cytotoxic materials from the cell. A revolutionary change in the perception of EVs/exosomes occurred when Valadi et al. (2007) reported the presence of nucleic acids in the lumen of exosomes. Additional studies suggested that EVs can also carry these bioactive molecules and deliver them to recipient cells, thereby perturbing the physiological status of recipient cells. This novel characteristic received a further boost when the 2013 Nobel Prize in Physiology or Medicine was awarded to James Rothman, Randy Schekman, and Thomas Sudhof for their contributions to vesicle study and demonstrating the pivotal role of EVs in cellular physiology. These seminal discoveries led to a paradigm shift in the status of exosomes from trash carrier to a new mode of intercellular communication. Researchers working in various fields have shown interest in EVs. Cancer biology is one such area of research that is dealing with the unique puzzle related to the transformation of normal cells to highly skilled immortalized cancer cells that take over the cells' normal physiology and the unique mechanism of disease spread called "metastasis." Since exosomes, or EVs, reached the cornerstone of cell-to-cell communication and perturbation of the cellular microenvironment, they have offered researchers a unique opportunity to study and understand this enigmatic disease and exceptional cellular phenomenon.

The area of EV research is expanding. We performed a survey of PubMed and observed an exponential increase in EV publications in the last decade (Figure 2a,b).

Studies conducted during the last 10 years have not only enhanced the understanding of the biological and functional role of exosomes/EVs, but have also increased the development of novel applications (Bunggulawa et al., 2018; Hood & Wickline, 2012). In this review, we bring together developments in EV research over the last decade, discuss how these developments have influenced the research landscape, identify the remaining challenges, and describe novel technologies being explored to overcome these challenges. In the years ahead, EV research will be propelled to the next stage of scientific discoveries and applications.

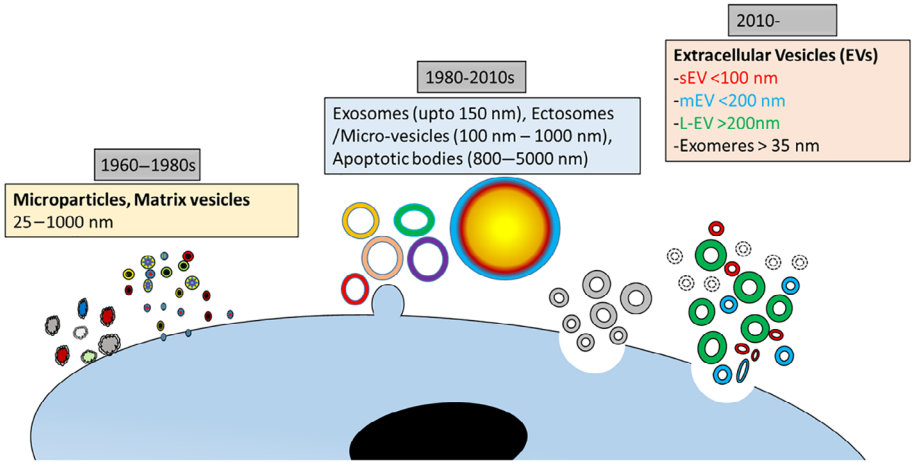

FIGURE 1 The evolution of nomenclature of extracellular vesicles (EVs) over last the few decades. With the discovery of vesicles in the 1960s, they were named based on the cell of origin. In 1980s, EVs were named "Exosomes," Micro-vesicles, and Apoptotic bodies, depending on the mechanism of biogenesis. Recently, a more streamlined nomenclature system was developed which subdivided EVs based on their size and origin. sEV, small extracellular vesicles; mEV, medium extracellular vesicles; L-EV, large extracellular vesicles

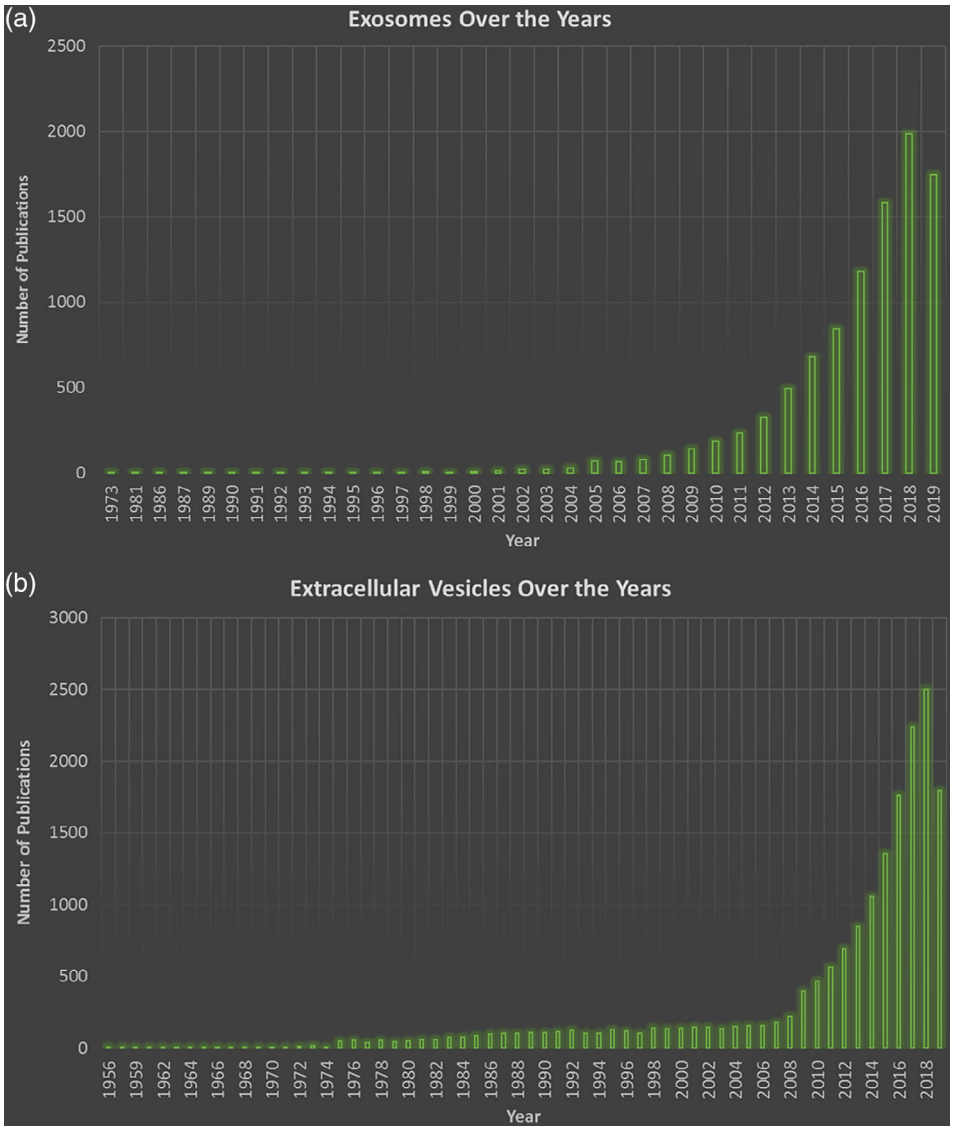

FIGURE 2 A dramatic surge in A, exosome and B, extracellular vesicle (EV)-based studies was observed in the last decade compared with the years following the discovery of EVs. This surge is another indicator of the significance and recognition of EVs by the scientific community in the last ten years

1.1 | EVs: Definition and biogenesis

EVs is a broad term used for various kinds of vesicles produced by the cells. Since the discovery of EVs in the 1960s, the names and definition of vesicles have seen many changes (Figure 3).

The most accepted definition is based on the vesicle size and mechanism of production. Vesicles of diameter <150 nm produced by differentiation of late endosomes to multivesicular bodies followed by their release from cells are called "exosomes." Also present is another set of vesicles that are up to 1,000 nm in diameter and produced by random budding from the plasma membrane without the involvement of any defined mechanism. Such vesicles are known as microvesicles (MVs), microparticles, apoptotic bodies, and oncosomes (Margolis & Sadovsky, 2019; Ortiz, 2017; Zaborowski, Balaj, Breakefield, & Lai, 2015). Interestingly, all except for apoptotic bodies can carry biomolecules in their lumens. Many studies have shown that these vesicles can bring about changes in their recipient cells and the surrounding microenvironment. However, in the literature, these definitions often overlap. Hence, in this review, we will use the term EV to refer to exosomes, as EV is a more acceptable name that includes all vesicles produced by the cells.

Recently, new technologies developed for imaging exosomes and EV isolation have made researchers aware of the amazing heterogeneous populations of EVs (Chiang & Chen, 2019; Willms, Cabañas, Mäger, Wood, & Vader, 2018). Zhang et al. (2018) recently discovered a new population of sub-nano-sized (<35 nm) non-membranous structures and named them "exomeres." Théry et al. (2018) came up with a more pragmatic nomenclature scheme. They defined vesicles <100 nm as small EVs, <200 nm as medium EVs, and >200 nm as large EVs. EVs are known to carry

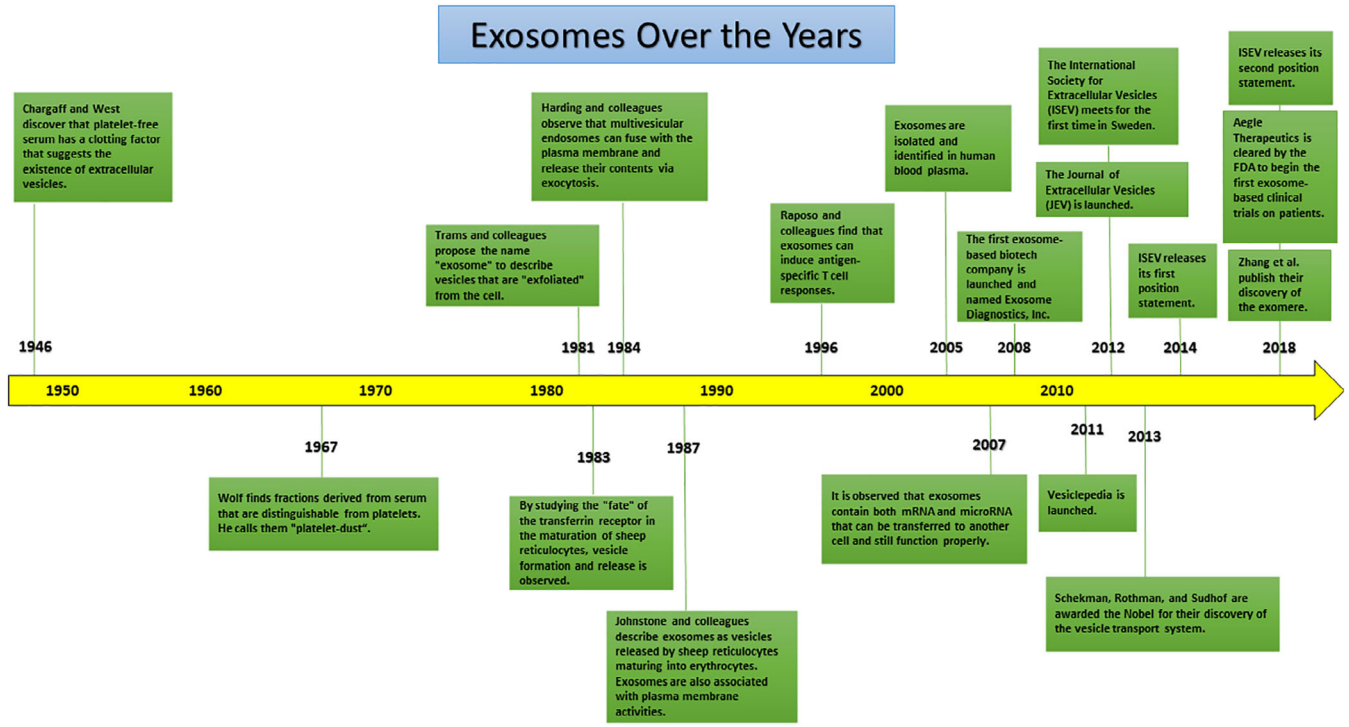

FIGURE 3 The development of extracellular vesicle since their discovery. The line bars show rapid discoveries from a number of studies conducted in the last decade

biomolecules, including several kinds of proteins, lipids, and nucleic acids, such as DNA, mRNAs, and noncoding RNAs like microRNAs (miRNAs) and long noncoding RNAs (Valadi et al., 2007; Zaborowski et al., 2015). These contents of EVs are biologically functional and reflect the composition of the cells of origin (Jella et al., 2018). This property has been explored extensively for its putative role in several physiological and pathological processes. In addition, the role of EVs has been of interest in translational medicine as a source for liquid biopsies, as EVs in bodily fluids carry a number of miRNA and proteins that have the potential to serve as novel diagnostic or prognostic biomarkers (Huang & Deng, 2019). Given the rapid research progression of EV biology, we herein provide an updated profile of the state-of-the-art advancements in this promising field, with a major focus on recent discoveries regarding the key activities of EVs, such as acquired resistance of cancer cells driven by exosomes in the tumor microenvironment (TME) (Figure 4).

2 | ADVANTAGES OF EVs FOR USE IN CANCER EXPERIMENTAL BIOLOGY

Exosomes or EVs offer new insight into cancer biology and have both diagnostic and therapeutic implications. Because of their cell-to-cell communication, EVs influence tumor progression, metastasis, and therapeutic efficacy. Studies have shown that EVs are present in all bodily fluids and are a rich source of biomarkers that can indicate the status of any pathological condition, including cancer (Brinton, Sloane, Kester, & Kelly, 2014; Li et al., 2017). In addition, the content of EVs can be engineered to reprogram the cancer-related properties (Fu et al., 2018). EVs produced by the tumors cells can perturb the TME to create favorable conditions for tumor progression, such as induction of the angiogenic switch, increased vascular permeability, and instigation of immune escape (Han, Zhao, Jiang, Yin, & Zhang, 2019; Wu, Wang, Hu, Yao, & Yu, 2019; Zimta, Baru, Badea, Buduru, & Berindan-Neagoe, 2019). In addition to enunciation of primary tumor-forming activities, EVs from tumor cells have been found to aid metastasis by creating a pre-metastatic niche and promoting epithelial-to-mesenchymal transition (Feng, Dean, Hornicek, Shi, & Duan, 2019; Guo et al., 2019; Vella, 2014). Furthermore, EVs exhibit chemo-protective properties for tumor cells and prevent any cytotoxic effect exerted by the administered chemo-therapeutic agent. In this way, tumor EVs can also aid in developing resistance to chemotherapy (Fu et al., 2018). Thus, tumor-derived EVs are essential to enhancing the lethality of cancer pathology. A further understanding of EV structure biogenesis and its functional importance in the context of cancer pathophysiology is needed.

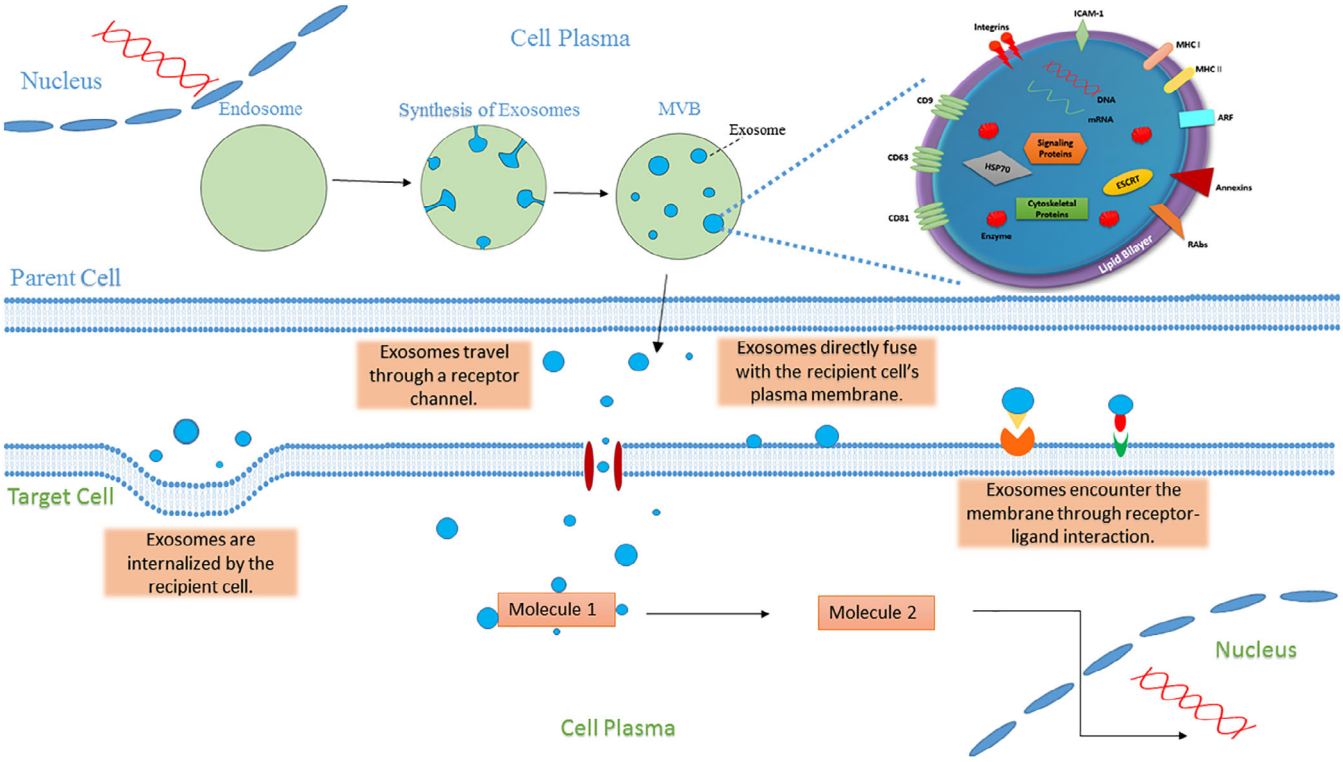

FIGURE 4 The biogenesis and biological role of various types of extracellular vesicles in cell-to-cell communication

In the last decade, EVs have influenced various facets of cancer research. Extensive studies on the role of EVs in cancer pathophysiology, including examinations of the TME, proliferation, angiogenesis, metastasis, resistance, and cancer immunology, have been conducted. Exciting diagnostic research has explored the development of diagnostic, prognostic, and predictive biomarkers. The following subsections discuss recently published studies that have furthered the understanding of cancer physiology and applications to a point at which EVs offer a new and pragmatic approach to solving this complex biological puzzle.

2.1 | EVs and cancer biology

With the advent of newer technologies and better understanding of EV biology and characteristics, many researchers have helped to improve our knowledge of processes related to cancer onset and progression. The initial studies were focused on the cargo content of EVs. In melanoma, EVs (exosomes) contribute to disease progression by contributing significantly to intercellular and intracellular communication, that is, between melanoma cells themselves, and between melanoma cells and neighboring normal cells, such as fibroblasts and endothelial cells. This process usually occurs via transfer of small noncoding RNAs, such as miRNA, packaged into the lumen. miR-222 is part of the exosomal cargo of melanoma cells. EVs released by miR-222-overexpressing cells are taken up by recipient primary melanoma cells to promote tumor growth through the activation of the PI3K/AKT pathway and downregulation of the tumor suppressor p27. Melanoma-cell-derived EVs promote the migration of endothelial cells and affect angiogenesis by transferring miR-9 to endothelial cells and activating the JAK-STAT pathway (Gajos-Michniewicz & Czyz, 2019).

Metastasis is a unique phenomenon that involves intense communication between cancer cells and their environment. Although no proper understanding of the mechanism of metastasis has been developed, various hypotheses are currently being evaluated. Until recently, when the status of EVs, in particular exosomes, was raised from that of a cellular waste carrier to an important mode of intercellular communication, several studies showed the role of EVs in various aspects of cancer metastasis. Cellular invasiveness is one of the earliest steps in metastasis. A study by McCready, Sims, Chan, and Jay (2010) showed that hsp90α present in the cargo of exosomes causes activation of tissue plasminogen activator MMP-2 and prompts invasiveness. Several studies of different cancers identified the role of exosome content in cancer progression (Kim, Shin, Kim, & Lee, 2019; Liu et al., 2016; Plebanek et al., 2017; Yousefi et al., 2019).

Interested readers are encouraged to read two reviews (Azmi, Bao, & Sarkar, 2013; Wortzel, Dror, Kenific, & Lyden, 2019) to obtain detailed information about the influence of exosomal content on cancer progression.

Three findings are considered the most important of the decade and have brought critical changes to the perception of the role of exosomes in cancer biology. The first report was published by Melo et al. (2014). Their findings suggested that, contrary to previous beliefs, exosomes were not "inert" cellular entities; rather, exosomes emanating from breast cancer (BC) cells carried a RNA induced silencing complex-loading complex that could process immature miRNAs to mature miRNAs, independent of the cellular machinery, indicating their functional activity. Although further studies are warranted, the initial finding is sufficient to suggest that exosomes or EVs have a pivotal role in the establishment of metastasis. One of the essential steps in metastasis is to develop a pre-metastatic niche that primes the future metastatic site with a favorable microenvironment so that metastatic cancer cells when they arrive, can establish themselves easily. The role of EVs in the development of the pre-metastatic niche has been recognized in many recent studies of colorectal, ovarian, and pancreatic cancer (Costa-Silva et al., 2015; Feng et al., 2019; Zeng et al., 2018).

Next, Hoshino et al. (2015) delineated the relationship between the homing property of exosomes/EVs and the organ specificity of a cancer cell type for forming metastatic sites. The findings demonstrated that the integrins present on tumor-derived EVs dictate the organotropic movement of metastatic cells. This study yielded a new direction for the theory of metastasis that significantly differed from Stephen Paget's seed-and-soild hypothesis established in 1889.

The latest significant discovery was a result of debate about the heterogeneous nature of EVs. Using Raman spectroscopy, Smith et al. (2015) demonstrated the existence of difference in composition of content of a subpopulation of EVs in bulk isolates. The difference in content suggested a distribution in EV functionality and their role in carcinogenesis (Zhang et al., 2018).

Research performed in the last decade has contributed significantly to our understanding of the role of EVs in cancer. The aforementioned discoveries have opened a new area of research that will hopefully result in the identification of novel pathways and therapeutic targets.

2.2 | EVs in cancer diagnostics

In addition to the role of EVs in cancer biology, researchers have realized the potential of EVs that can stably carry biomolecules (Thakur et al., 2014; Thind & Wilson, 2016; Xiao et al., 2012). DNA, several species of noncoding and coding RNAs, and proteins have been exploited to develop biomarkers for early diagnosis and to predict prognosis or response to therapy. For example, DNA, RNA, and proteins stably encapsulated in exosomes/EVs can be exploited to diagnose melanoma at an early stage (Xiao et al., 2012). Similarly in lung, breast, colorectal, and prostate cancer, the molecular composition of EVs has shown promising opportunities to develop diagnostic, prognostic, and predictive biomarkers (Bracht, Mayo-de-las-Casas, Berenguer, Karachaliou, & Rosell, 2018; König et al., 2017; Øverbye et al., 2015; Tovar-Camargo, Toden, & Goel, 2016). In one study of lung cancer, miRNAs isolated from exosomes showed high reliability in their utility as putative biomarkers, as they were able to distinguish between healthy individuals and lung cancer patients with a sensitivity and specificity of 80.3% and 92.3%, respectively (Wu, Yang, Dai, Zhu, & Chen, 2019).

Several isolation techniques developed during the past decade have aided in isolation of EVs from various bodily fluids. This work resulted in the development of robust liquid biopsy modalities, which are minimal or noninvasive in nature and offer minimal discomfort (Cazzoli et al., 2013; Srivastava et al., 2018; Whiteside, 2015). In another study, Thakur et al. (2014) showed that epidermal growth factor receptor mutations can be detected in exosomal DNA of four non-small cell lung cancer (NSCLC) cell lines, suggesting that exosomal DNA, RNA, and proteins can be used to detect mutations, gene fusions, and splice variants in NSCLC. With the development of novel exosome isolation strategies (discussed later), various bodily fluids have been actively explored for use in diagnostics.

2.3 | EVs in cancer therapeutics

The ability of EVs to carry and transport biomolecules from donor to recipient cells prompted researchers to explore EVs as drug delivery vehicles (Batrakova & Kim, 2015; Kibria, Ramos, Wan, Gius, & Liu, 2018; Lakhal & Wood, 2011). Further, being similar in structure and composition, EVs have evolved as an alternative option for conventional lipid drug carriers for delivery of therapeutics. In the last few years, there has been an increase in research devising

innovative strategies for loading drugs and anti-cancer molecules into exosomes. In the following section, some of these recently developed loading techniques are discussed.

2.3.1 | Physical loading of EVs

Exosomes possess an outer double-membrane composed of lipoproteins. The membrane structure is in a dynamic state both at room temperature and at 37°C, that faclitates passive uptake of molecules (therapeutic cargo). This method allows loading of both, hydrophobic and hydrophilic anticancer drugs and small molecules. Various approaches employed for loading therapeutics onto exosomes are described below.

2.3.2 | Incubation with EVs

In this method, EVs are isolated from cells and then loaded with therapeutic cargo. Cargo to be loaded is incubated with exosomes and diffuses inside the EVs through a concentration gradient formed across the dynamic exosome membranes. Drugs and small molecule inhibitors are commonly loaded using this procedure. Sun et al. (2010) reported loading of EL4 exosomes with curcumin in Phosphate buffered saline by incubating at 22°C for 5 min. The mixture of curcumin-containing and free exosomes was then purified by gradient ultracentrifugation (UC) step that separated free curcumin, free exosomes, and exosome-curcumin complex in three different gradients based on their density. Upon application, the exosomes-curcumin complex showed a superior outcome compared with conventional lipid-based delivery methods. This better outcome was attributed to the complex being protected from lipopolysaccharide-induced septic shock and uptake by activated monocyte-derived myeloid cells circulating in the peripheral blood. These exosome-curcumin complexes showed significant *in vitro* and *in vivo* therapeutic efficacy.

As an alternate approach, small molecule inhibitors and chemotherapeutic drugs can also be incubated with EVs using organic solvents. Munagala, Aqil, Jeyabalan, and Gupta (2016) reported successful loading of bovine milk derived exosome with chemopreventive agents (withaferin A, bilberry-derived anthocyanidins, and curcumin), and chemotherapeutic (paclitaxel and docetaxel) agents, in a 1:1 ratio of ethanol and acetonitrile solvents and purified by centrifugation. They found that the presence up to 10% of solvents did not affect the physical properties and therapeutic efficacy of the drug containing exosomes against cancer cell lines.

2.3.3 | Incubation with donor cells (passive loading)

In passive loading, therapeutic cargos are added to donor (fibroblast, macrophage, dendritic, etc.) cells which package their EVs with the added therapeutic cargos in the process of EV biogenesis. This method has mostly been used with biomolecules, such as miRNA, mRNA, proteins, and DNAs of therapeutic significance. Mathiyalagan and Sahoo (2017) developed the method for encapsulating Cy3 dye-labeled pre-miR miRNA precursors secreted exosomes to human CD34+ stem cells. First, they transfected the cells with commercial transfecting agent lipofectamine that was encapsulated with Cy3 dye-labeled pre-miR miRNA precursors. After 24 h, they isolated and purified the EVs secreted with Cy3 dye-labeled pre-miR miRNA and showed better quality of labeled EVs, which was confirmed by flow analysis. Then, they co-transfected the isolated EVs miRNA complex in human umbilical vein endothelial cells.

Pascucci et al. (2014) incubated the anti-cancer drug paclitaxel with SR4987 mesenchymal stromal cells (MSCs). They then isolated the MVs by UC and estimated the secretion of paclitaxel concentration in MVs, ultimately finding that a significant amount of paclitaxel was packaged in the MVs. The MVs that secreted paclitaxel demonstrated higher anti-proliferative activity in the CFPAC-1 human pancreatic cell line.

2.3.4 | Electroporation

In electroporation, EVs are mixed with the therapeutic cargo of interest, and different volts of electric fields are externally applied. The electric field creates pores by disturbing the EV membrane, and the pores allow the therapeutic cargos to diffuse inside the EV (exosome) membrane. After removing the electric field, the exosome membrane recovers

its structure. Since large molecules tend to form aggregates with EVs, this method is the most useful for loading large molecular weight therapeutic cargos, such as siRNA, mRNA, DNA, and miRNA, into EVs.

Asadirad et al. (2019) reported that different concentrations of miRNA-155 on loading into CT26 tumor cell-derived exosomes by electroporation with 0.100, 0.200, and 0.300 kV altered the size and shape of the exosomes. These miRNA-155-loaded exosomes were successfully delivered into dendritic cells and showed increased surface expression levels of MHCII (I/A-I/E), CD86, CD40, and CD83. Another group reported loading of siRNA onto exosomes derived from human embryonic kidney (HEK-293) cells by electroporation with an exosomes:siRNA molar ratio of 1:60. The siRNA loading efficiency was around 20% and could deliver the siRNA-loaded exosomes into PANC-1 cancer cells. These exosomes loaded with siRNA showed better cell uptake and therapeutic efficacy *in vitro* (Faruqu, Xu, & Al-Jamal, 2018).

2.3.5 | Sonication

Sonication, in general is a harsh method which sometimes results in irreversible disruption of the cellular membrane. In this procedure, a probe sonicator disrupts the exosome membrane structure that facliates loading of therapeutics rapidly. After sonication is complete, the membrane structure returns to its original arrangement resulting in entrapment of the drug inside the exosome. This approach is useful for loading drugs that are highly hydrophobic and pose challenge in clinical practice.

Liu et al. (2019) reported that the poorly water-soluble anti-cancer drug triptolide was loaded onto SKOV3 ovarian cancer cells-derived exosomes by sonication using 20% power, 2 s pulse, and 2 s pause in 15 total cycles. They reported high drug loading efficiency and prononuced antitumor activity both, *in vitro* and *in vivo*. The drawback of this formulation however, was liver and spleen toxicity.

Sonication method has also been applied for loading anticancer drugs onto M1-macrophages-derived exosomes. Hydrophobic anti-cancer drug, paclitaxel was loaded on M1-macrophages-derived exosomes by sonication with 20% amplitude, 30 s pulse, and 30 s pause for a total of 6 cycles. This paclitaxel loaded on to M1 exosomes showed improved *in vitro* and *in vivo* therapeutic efficacy than did free paclitaxel and M1 exosomes in mice bearing BC cell tumors (Wang et al., 2019).

2.3.6 | Freeze/thaw method

In the freeze/thaw procedure, therapeutic cargo is mixed with EVs, incubated at room temperature for a fixed time, quickly frozen at either −80°C or with liquid nitrogen, and then thawed at room temperature. The same procedure is repeated three times to confirm cargo encapsulation. This procedure tends to produce EV aggregations and leads to EVs of larger size. EV (exosome) membranes possess a lipid bilayer structure that usually interacts with various lipid membrane structures and fuses within the EV structures. Recently, Sato et al. (2016) fused liposomes labeled with fluorescent dyes (1 mol% 1,2-dinyristoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) and rho-DMPE) with exosome membranes by repeatedly freezing these mixtures with liquid nitrogen and thawing at room temperature. Later, fluorescence resonance energy transfer analysis confirmed the fusion efficiency of 1,2-dinyristoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl; NBD-DMPE) and rhodamine (rho)-DMPE with exosomes.

2.3.7 | Extrusion

In this method, therapeutic cargos are mixed with EVs and extruded with a lipid syringe extruder with pore size between 100 and 400 nm. During this process, the EV membrane opens up and mixes with drug. Extrusion yields changes in membrane reformation, which changes the EVs' physical properties. Kalimuthu et al. (2018) used exosomes derived from MSCs, called exosome mimetics (EMs), to load the anti-cancer drug paclitaxel onto the EMs, isolated from human bone marrow-derived MSCs by extrusion. Different concentrations of paclitaxel were mixed with EMs and extruded with different sizes of membrane filters (1, 5, and 10 mm). These paclitaxel-loaded EMs showed increased cell killing efficiency *in vitro* and tumor growth inhibition compared with controls and individual groups in MBA-MB-231 breast cancer (BC) cell line models.

Another group loaded the antioxidant catalase onto exosomes through extrusion, sonication, incubation, and freeze–thaw for Parkinson's disease (PD) treatment. They found that extrusion and sonication yielded high loading efficiency, sustained release, and high catalase activity. These delivery methods produced promising *in vitro* and *in vivo* treatment effects for inflammatory and neurodegenerative disorders (Haney et al., 2015).

2.3.8 | Chemical conjugation

The surfaces of EVs can be conjugated with drug molecules and targeting ligands through chemical methods by using crosslinkers. Click chemistry, a cycloaddition reaction conjugated by the azide and alkyne functional groups to form triazole linkages, is one of the most commonly used methods. This method can be used for conjugations of small- and macro-molecules.

1-Ethyl-3(3-dimethylaminopropyl) CarbodiimideN-hydroxysuccinimide and maleimide-thiol reactions are also used for conjugation of small and macromolecules on the surfaces of EVs. These two chemical reactions can be carried out in an aqueous environment. Jia et al. (2018) conjugated the RGERPPR (RGE)-peptide on the surface of EVs for receptor targeting in a two-step process. Initially, they conjugated an alkyne group into the exosome membrane through an EDC/NHS reaction, followed by conjugation with RGE-peptide, which contains an azide functional group in the presence of Cu-catalysis. They used the electroporation method to load Superparamagnetic iron oxide nanoparticles and curcumin into exosomes for imaging and therapeutic applications. These RGE-peptide-conjugated exosomes showed promising *in vitro* and *in vivo* therapeutic and imaging results, compared with non-targeted and individual treatments. Another group also reported using the click chemistry method for conjugating glycon/glycoproteins (amino acids and saccharides) for metabolic insertions on the surface of exosomes. They used B6F10-derived exosomes to conjugate L-azidohomoalanine for metabolic insertions (Wang, Altinoglu, Takeda, & Xu, 2015).

2.3.9 | Nanoparticle-drug complexes

Nanoparticle-drug complexes are formed by conjugating drug/gene molecules with metallic-based nanoparticles via different chemical strategies. These nanoparticles are then loaded onto the EVs by any of the above mentioned methodologies. The advantage of nanoparticle-drug complexes is increased sustained drug release, because drug must cleave from nanoparticles and diffuse out of the EVs to initiate its therapeutic effect, whereas in the above mentioned methods, only one step is needed to diffuse drug out of the EVs. Nanoparticles can provide additional advantages, such as imaging properties. Self-therapeutic and drug conjugations to the nanoparticles can be made for tumor microenvironment-cleavable linkages to reduce the toxicity to normal tissues. Our group recently published a report using nanosomes for doxorubicin delivery. We conjugated the anti-cancer drug doxorubicin to the gold nanoparticles through a pH-sensitive, acidic tumor microenvironment cleavable linker and loaded it onto exosomes derived from MRC9 fibroblast cells. These drug-nanoparticle-loaded EVs showed better therapeutic efficiency in non-small cell lung cancer cell lines and reduced toxicity against normal lung fibroblasts and cardiomyocytes (Srivastava et al., 2016).

3 | EVS IN CLINICAL PRACTICE

As described previously, EVs/exosomes have multiple functions in human carcinogenesis and are involved in tumor progression, invasion, metastasis, angiogenesis, escaping the immune system, and drug resistance (Whiteside, 2015). Tumor-specific circulating EV miRNAs can be used as diagnostic and prognostic markers. They are stable at extreme temperatures and, due to their lipid bilayer, are protected from degradation. These features make them ideal as biomarkers and vehicles for drug delivery (Kosaka, Iguchi, & Ochiya, 2010).

Future research regarding exosomes is predicted to have a two-fold focus: (a) their application as nanocarriers during the development of novel targeted therapies and (b) the assessment of their ability to provide accurate information for the formulation of a specific cancer diagnosis or prognosis. In the next sections, we will discuss the role of EVs in the most common cancers worldwide: breast and lung cancer. Significant work has been accomplished with respect to EVs as diagnostic and prognostic markers.

3.1 | Breast cancer

Breast cancer (BC) is the leading cause of cancer death in women worldwide. In the United States, BC is the most common female cancer and the second most common cause of cancer death (Siegel, Miller, & Jemal, 2019). Gene array technology has led to the recognition that BC is a heterogeneous disease composed of different biological subtypes. Genetic profiling enables us to predict the response to chemotherapy (Libson & Lippman, 2014).

Hannafon et al. (2016) reported that levels of miR-21 and miR-1246 were detected at significantly higher levels in the plasma exosomes of 16 patients with BC than in the plasma exosomes of healthy controls. They profiled exosomal miRs from a normal mammary epithelial cell line (MCF10A) and multiple breast carcinoma lines (MCF7 and MDA231) and showed that miR-1246 was enriched in tumor-derived exosomes. More recently, Li et al. (2018) published a study of 400 plasma samples that concluded that miR-106a-5p and miR-20b-5p, which are a part of the miR-106a-363 cluster on chromosome X, are extremely promising biomarkers for BC diagnosis.

Triple-negative BC (TNBC) is a disease with poor prognosis. Eichelser et al. (2014) showed that exosomal miR-373 in the serum was significantly increased in patients with TNBC compared with those with luminal tumors or healthy controls. They also found that overexpression of miR-373 in MCF-7 cells showed downregulated protein expression of the estrogen receptor, and inhibited apoptosis induced by camptothecin.

Exosomal RNAs are also under investigation as a potential causative factor for resistance to chemotherapy and/or hormone therapy in BC. Drug resistance in BC cells results from different mechanisms. Among these mechanisms, resistance mediated by exosomes and exosomal miRNAs has attracted recent attention. There is growing evidence of the emerging roles of miRNAs in the resistance of BC cells to therapeutic agents. Chen et al. (2014) added exosomes from adriamycin- and doxorubicin-resistant BC cells to the culture medium of wild-type (WT) BC cells. Chemosensitive cells became chemo-resistant due to the delivery of multidrug resistance-associated microRNAs: miR-100, miR-222, miR-30a, and miR-17. Similar findings were noted when tamoxifen-resistant MCF-7 was added to WT MCF-7 cells. The resistant cells released exosomes loaded with miR-221 and miR-222, which caused the inhibition of p27 and ERα mRNA translation in WT MCF-7 cells, rendering the cells hormone-resistant (Liang et al., 2018). In another experiment, miR16 was shown to be upregulated in plasma exosomes from BC patients with the recurrent disease compared with healthy donors. Downregulation of miR-30b was observed in plasma exosomes from BC patients with recurrence (Ni et al., 2018).

3.2 | Lung cancer

Every year, 1.8 million people are diagnosed with lung cancer and 1.6 million die of the disease (Ferlay et al., 2014). Approximately 85% of patients fall into the group of histological subtype known as non-small cell lung carcinoma (NSCLC), of which lung adenocarcinoma and lung squamous cell carcinoma are the most common subtypes (Molina, Yang, Cassivi, Schild, & Adjei, 2008). In recent years, several predictive biomarkers were studied as companion tests for numerous oncologic compounds. Biomarkers can predict treatment activity and reduce costs while obviating potential harms of unnecessary treatment. The mutation of human EGFR is a successful example in the treatment of NSCLC (Patel, Ersek, & Kim, 2015). The expression pattern of programmed death-ligand (PDL)1 protein on tumor cells was suggested as a potential predictive biomarker because it is implicated in the mechanism of action of immune checkpoint inhibitors; however, controversial and sometimes conflicting results have been reported. Exosomes and exosomal RNA can be considered ideal biomarkers for cancer diagnosis, prognosis, and targeted therapy because they closely resemble the condition of parent cells and can be easily collected from patients.

The authors realize that conventional diagnostic modalities like tissue biopsies will remain an integral part of NSCLC standard of care. However, recent advancements in research has opened new avenues to explore the utility of exosomes in clinical diagnosis. Recent studies have shown that both EV miRNA and proteins can be used as biomarkers for early diagnosis of NSCLC in asymptomatic patients (Clark, Fondrie, Yang, & Mao, 2016; Jakobsen et al., 2015; Markou, Sourvinou, Vorkas, Yousef, & Lianidou, 2013). Bianchi et al. (2011) provided a 34-miRNA panel that can be used in high-risk asymptomatic patients to diagnose NSCLC with 80% accuracy.

Currently, there is no blood-based tumor marker or biomarker for monitoring NSCLC therapy. Patients undergoing systemic therapy are monitored by computed tomograph (CT) scans at 9–12 week intervals. These intervals can be a long time for a patient who is not responding. In one study, the mutation of EGFR T790M in plasma from NSCLC patients was detected with higher sensitivity (92%) and specificity (89%) in a combination of exosomal RNA/DNA and

circulating free tumor DNA (cfDNA) than in cfDNA alone (Castellanos-Rizaldos et al., 2019). Measurement/clearance of these EV markers can be monitored during therapy to predict response and prognosis, similar to how prostate-specific antigen (PSA) is used in prostate cancer carcinoembryonic antigen (CEA) is used in gastrointestinal cancer and alpha-fetoprotein (AFP) testing is used in hepatocellular cancer (Sharma, 2009). In another study of 226 patients with NSCLC, high serum miR-19a expression was found to be an independent poor prognostic factor. Survival analysis revealed that the overall survival rate of patients with high serum miR-19a expression was significantly worse than that of patients with low serum miR-19a expression (Lin et al., 2013). Kanaoka et al. (2018) showed in the miRNA microarray analysis that exosomal miR-451a was upregulated in NSCLC patients with recurrence and was significantly associated with lymph node metastasis, vascular invasion, clinical stages, and survival status of NSCLC patients.

4 | CURRENT STATUS OF EV RESEARCH

The past decade of EV research has established EVs as integral participants in cancer and other diseases. Concerns have begun to surface over issues of purification of homogenous populations of EVs, reproducibility of results, and availability of convenient methods, all of which are required to translate results from bench to bedside (Tang, Lv, Lan, & Liu, 2019; Xu, Greening, Zhu, Takahashi, & Simpson, 2016). These concerns are related to the absence of a universally accepted and robust protocol or method for isolation of EVs from cell culture and bodily fluids. Realizing the importance of rigor and convenience, several recent studies have been conducted to develop novel and interesting approaches to isolate and concentrate GMP-grade EVs from various sources (Boukouris & Mathivanan, 2015; Gurunathan, Kang, Jeyaraj, Qasim, & Kim, 2019).

As before, this aspect of EV research presents an excellent example of interdisciplinary research in which researchers from diverse backgrounds worked synergistically to resolve the issue. Yu et al. (2018) published an interesting review on EV isolation in 2018. They described various strategies for EV isolation and purification. They characterized UC, density gradient centrifugation (DGC), size exclusion chromatography (SEC), and reagent-based methods as conventional methods of isolation and compared these methods with some newly developed strategies. Théry et al. published an UC-based method of isolation of EVs (exosomes) in 2006. To date, UC is the most popular method among EV researchers, although various studies have reported associated drawbacks. The major concerns are the effect of strong G forces acting on the EVs, co-precipitation of several insoluble proteins, and high polydispersity in the isolates. UC is also a labor intensive, time-consuming process that requires specialized training and instruments (Chen et al., 2019; Linares, Tan, Gounou, Arraud, & Brisson, 2015; Théry, Amigorena, Raposo, & Clayton, 2006). Some of these drawbacks were acknowledged by Théry et al. in their 2017 article (Tkach, Kowal, & Théry, 2017). In addition to UC, several other methods based on SEC and DGC were developed (Guerreiro et al., 2018; Konoshenko, Lekchnov, Vlassov, & Laktionov, 2018; Takov, Yellon, & Davidson, 2018). Around the same time, System Bioscience, Inc., a California-based exosome research company, developed ExoQuick, an exosome precipitation reagent. ExoQuick was probably the first commercial EV-related product in the market. This reagent consisted of proprietary material that can pull down exosomes from a small volume and complex materials, such as serum and urine, in a short time using simple centrifugation steps. ExoQuick is still a popular product among EV researchers, especially those who are beginning to foray into this arena of research. The popularity of ExoQuick inspired many other companies to develop similar products, such as Total Exosome Isolation Reagent from Invitrogen/ThermoFisher Scientific, ExoEasy from Qiagen, and the miRCURY Exosome kit from Exicon (now Qiagen).

Rigorous research has been conducted to develop novel methods to circumvent the disadvantages associated with conventional methods. In the following section, we describe some of the latest research and interesting technology developed in the area of EV isolation and purification.

4.1 | Novel approaches and technologies developed for isolation and purification of EVs

In addition to the traditional UC methods, alternative strategies that favor either quicker or purer yield of the EVs have been developed. One such method is “optimized ultrafiltration”, in which a 0.22-μm filter is used, followed by using a dialysis membrane with the molecular weight cut off of 10 kDa. This method improved the quality, number of EVs, and biological safety of the EVs obtained from critical samples, such as urine samples, compared with the UC method (He, Zhu, Wang, & Wu, 2019). Another novel EV isolation method uses affinity-based enrichment with TIM4 protein.

The protein specifically binds to the phosphatidylserine molecules present on the surface of the EVs. Since the binding of TIM4 to phosphatidylserine can be released using Ca^{2+} addition, it is easier to obtain intact EVs through this method. It has also been shown to result in the identification of more EV-specific proteins through mass spectroscopy (Nakai et al., 2016). The addition of one-step sucrose cushion UC, has been shown to improve the yield and quality of exosome isolations (Gupta et al., 2018). An additional strategy consisting of an automated process of EV isolation using UC through a device called Exodisc-B has recently been developed. This device is made of two inbuilt chambers that separate larger particles and enrich the EVs in 40 min. The device is efficient in preparing the EVs from a volume as low as 30 μL, with a recovery efficiency of >75% (Sunkara et al., 2019).

UC methods are cumbersome. Hence, the affinity of antigen and antibody has been recently exploited to develop small-scale isolation strategies. The innovative strategy of nanowires conjugated with a cocktail of antibodies yielded an efficient purification of a homogenous population of exosomes from a smaller volume of samples with improved uniformity and threefold higher yield compared with that of traditional methods (Lim et al., 2019). The use of antibody-conjugated polypyrrole nanowires has also been shown to capture exosomes based on the marker protein. The exosomes are released and recovered from the nanowires via electrical or glutathione stimulation (Lim, Choi, Lee, Han, & Cho, 2019). Isolation of high-quality exosomes by utilizing the specific, but reversible, binding of TiO_2 to the phosphate groups of the exosome lipid bilayer is another potential method. This approach can be accomplished in as little as 5 min to obtain exosomes from human serum samples with a recovery rate of 93.4% (Gao et al., 2018). Immunocapture of the EVs is another strategy to isolate the exosomes with specific markers. Antibodies against general extracellular markers, such as CD9, CD63, and CD81, were used to capture EVs from urine (Campos-Silva et al., 2019).

The addition of a microfluidic device that uses surface protein markers to isolate exosomes is another incremental development in the purification of EVs. This method uses surface markers, such as CD63 and EpCAM, to enrich the exosomes that are free of contamination. This approach was used on high-grade serious ovarian cancer (HGSOC), and identified STAT3 and HGF proteins as being significantly regulated in HGSOC compared with ovarian surface epithelial cells and fallopian tube secretary epithelial cells (Dorayappan et al., 2019). One approach that described the use of 3D printed PDMS microfluidic devices for an integrated harvesting and photo-release of surface engineered exosomes with tumor antigens (gp-100, MART-1, MAGE-A3) has also been shown to be effective towards generating exosomes that are intact and functional in presenting antigen to T cells and inducing cytolysis, compared with the naive exosomes (Zhao, McGill, Gamero-Kubota, & He, 2019).

A label-free and contact-free method for the isolation of exosomes from blood using an integrated acoustics and microfluidics approach was described recently. This acoustofluidic device contains two modules: the first module removes the larger blood components and the second module extracts EV subgroups, resulting in 98.4% purity (Wu et al., 2017). Another modified version of a microfluidic device-based exosome isolation protocol uses a viscoelasticity-based microfluidic system that employs a label-free, continuous, and size-dependent separation method. This method resulted in high purity (>90%) and recovery (80%) of the exosomes (Liu et al., 2017). Takara Bio USA has developed a non-antibody column-based kit of exosome isolation called Capturem. This kit can isolate exosomes from cell culture medium within 30 min. Briefly, the precleared cell culture supernatant is loaded onto the column, followed by a spin at 1000*g* for 2–3 min at room temperature. The supernatant is discarded and intact exosomes bound to the column are washed and collected using wash buffer. This kit is promising largely because it is not antibody-based, and thus the isolation of exosomes is expected to be unbiased. However, the exact advantages of this kit over other commercially available isolation kits remain to be explored and established.

In another method, exosome production from cells was enhanced by using bare neutral and cationic liposomes. The delivered liposomes to stimulate cells, resulting in enhanced production of exosomes. Emam et al. (2018) discovered this property in cancer cells. However, its utility in isolation of exosomes from non-cancer cells and from the surface of cells remains to be investigated. A similar attempt to induce the production of exosomes by overexpressing exosome marker CD9 was shown to result in a high yield of exosomes. Although these studies were performed in the context of studying lentiviral efficacy, the concept holds promise for large-scale production of exosomes/EVs (Böker et al., 2018; Schiller, Lemus-Diaz, Rinaldi Ferreira, Böker, & Gruber, 2018).

5 | MOLECULE CONTENT OF EVs

The molecular content of EVs, specifically exosomes, has been of interest. As mentioned above, researchers interested in biomarker discovery and identification of therapeutic targets explored and reported the presence of biomolecules in

the lumen of exosomes. There was an immediate need to identify exosomal markers for characterization of exosomes in general and those originating from a specific cell or tissue type, for example, cancer-cell-derived exosomes.

Considering the importance and lack of such a resource, Mathivanan et al. developed an open source compendium of exosome molecular content called "ExoCarta" (Keerthikumar et al., 2016). They manually curated proteins, nucleic acids (miRNA/mRNA), and lipids based on the researchers' reports of exosomes isolated from different sources and species. In 2009, ExoCarta catalogued 2,399 proteins, 901 mRNAs, and 274 miRNAs identified through 64 studies. This database has helped a number of investigators. Further, as the number of studies has increased, more data have been deposited. After one decade, the current database has more than 9,000 proteins, 3,000 mRNA, and 2,500 miRNA reported from 286 studies (Table 1). This immense growth indicated the strong development of exosome and EV research during the past decade.

6 | FUTURE CHALLENGES AND CONCLUSION

Although several gaps remain, the past decade has seen exponential growth in the field of exosome research. Advanced EV-specific technologies have been developed and evaluated for isolation of EVs *in vitro* and *in vivo*. These advanced and high-precision methodologies have enabled researchers to deepen the understanding of the biology and application of EVs. However, some challenges must be resolved before this research advances. The first challenge regards the definition and nomenclature of EVs. Since the beginning, there has been an overlap in naming the EVs. The heterogeneity of exosomal populations has hindered our understanding of their biogenesis, molecular composition, biodistribution, and functions. Similarly, with the advent of EV-specific technologies, we now have more detailed knowledge about the cargo content of EVs, but our understanding of EV biology and biogenesis is limited. Knowledge of cargo loading will help in devising useful strategies and methods that can be used for human benefit. Additionally, scaling up uniform EV production must be addressed before EV-based tools, especially those related to therapeutic delivery, can progress. The current studies of those aspects of EVs are based on proof-of-concept. Methods to isolate EVs in bulk from limited materials, such as blood and other bodily fluids, must be developed.

The storage of isolated EVs is also a challenge. Independent researchers have tested the feasibility of storing of EVs at different temperatures for different time points; these studies have produced different outcomes. If EVs are considered as an enriched source of biomarkers and therapeutic carriers, efforts must be made to develop a uniform protocol for storing EVs. In our opinion, the next position paper on EVs characterization from international society of extracellular vesicles (ISEV) should suggest uniform guidelines for short- and long-term storage of EVs.

Nevertheless, exosome science has made significant strides. Currently, 28 clinical trials of exosomes and cancer are listed on the clinicaltrials.gov website (Table 2). The field of EV research has seen unprecedented growth in the last decade, and continues to grow, with researchers bringing novel approaches and methods. In the last decade, ISEV has published two position papers on EV characterization and nomenclature. This step was needed to streamline the EV research approach (for standardization of isolation and storage of EVs) and avoid confusion over the definition, classification, and nomenclature of EVs. These steps were intended to enable people from diverse research disciplines to perform systematic research on EVs. The last decade of EV research may be considered a developmental phase during which newer technologies aiding EV research were developed and some insightful knowledge was gathered about the basic biology of EVs. The future of EV research is exciting and promising.

TABLE 1 The molecular content described in ExoCarta, 2009–2019

Description	Year 2009[a]	Year 2019[b]
Number of exosome studies	64	286
Number of proteins	2,399	9,769
Number of mRNA	901	3,408
Number of miRNA	274	2,838
Number of lipids	—	1,116

[a]As described in Mathivanan and Simpson (2009).
[b]As on database last accessed on January 10, 2019.

TABLE 2 Current exosome-based clinical trials as reported on the www.clinicaltrials.gov website accessed on March 9, 2019

Title	Status	Conditions	Clinical Trial No.
Combined Diagnosis of CT and Exosome in Early Lung Cancer	Not yet recruiting	Early lung cancer	NCT03542253
Acquisition of Portal Venous CTCs and Exosomes From Patients With Pancreatic Cancer by EUS	Recruiting	Pancreatic cancer	NCT03821909
Circulating Exosomes As Potential Prognostic And Predictive Biomarkers In Advanced Gastric Cancer Patients ("EXO-PPP Study")	Unknown status	Gastric cancer	NCT01779583
Clinical Validation of a Urinary Exosome Gene Signature in Men Presenting for Suspicion of Prostate Cancer	Completed	Prostate cancer	NCT02702856
Interrogation of Exosome-mediated Intercellular Signaling in Patients With Pancreatic Cancer	Active, not recruiting	Pancreatic cancer, benign pancreatic disease	NCT02393703
Trial of a Vaccination With Tumor Antigen-loaded Dendritic Cell-derived Exosomes	Completed	Non-small cell lung cancer	NCT01159288
Study Investigating the Ability of Plant Exosomes to Deliver Curcumin to Normal and Colon Cancer Tissue	Active, not recruiting	Colon cancer	NCT01668849
Analyses of Exosomes in the Cerebrospinal Fluid for Breast Cancer Patients With Suspicion of Leptomeningeal Metastasis	Not yet recruiting	Breast cancer, leptomeningeal metastasis	NCT03974204
Edible Plant Exosome Ability to Prevent Oral Mucositis Associated With Chemoradiation Treatment of Head and Neck Cancer	Active, not recruiting	Head and neck cancer, oral mucositis	NCT01668849
A Pilot Study of Tumor-Derived Exosomes as Diagnostic and Prognostic Markers in Breast Cancer Patients Receiving Neoadjuvant Chemotherapy	Withdrawn	Breast neoplasms	NCT01344109
Non-coding RNA in the Exosome of the Epithelia Ovarian Cancer	Recruiting	High grade serous carcinoma, ovarian cancer, exosomes, prognosis, early diagnosis	NCT03738319
Exosomes in Rectal Cancer	Recruiting	Rectal cancer	NCT03874559
Metformin Hydrochloride in Affecting Cytokines and Exosomes in Patients With Head and Neck Cancer	Active, not recruiting	Larynx, lip, oral cavity, pharynx	NCT03109873
Exosome Testing as a Screening Modality for Human Papillomavirus-Positive Oropharyngeal Squamous Cell Carcinoma	Recruiting	Oropharyngeal cancer	NCT02147418

(Continues)

TABLE 2 (Continued)

Title	Status	Conditions	Clinical Trial No.
Diagnostic Accuracy of Circulating Tumor Cells (CTCs) and Onco-exosome Quantification in the Diagnosis of Pancreatic Cancer—PANC-CTC	Completed	Pancreatic ductal adenocarcinoma	NCT03032913
Clinical Research for the Consistency Analysis of PD-L1 in Cancer Tissue and Plasma Exosome	Unknown status	NSCLC	NCT02890849
Clinical Research for the Consistency Analysis of PD-L1 in Lung Cancer Tissue and Plasma Exosome Before and After Radiotherapy	Unknown status	NSCLC	NCT02869685
Exosomes Implication in PD1-PD-L1 Activation in OSAS	Not yet recruiting	Sleep apnea syndromes, obstructive, cancer	NCT03811600
iExosomes in Treating Participants With Metastatic Pancreas Cancer With KrasG12D Mutation	Not yet recruiting	KRAS NP_004976.2:p.G12D, metastatic pancreatic adenocarcinoma, pancreatic ductal adenocarcinoma, Stage IV pancreatic cancer AJCC v8	NCT03608631
Serum Exosomal Long Noncoding RNAs as Potential Biomarkers for Lung Cancer Diagnosis	Recruiting	Lung cancer (diagnosis)	NCT03830619
A Study of Circulating Exosome Proteomics In Gallbladder Carcinoma Patients	Recruiting	Proteinosis, gallbladder carcinoma	NCT03581435
Circulating Exosome RNA in Lung Metastases of Primary High-Grade Osteosarcoma	Recruiting	Lung metastases, osteosarcoma	NCT03108677
Study of Exosomes in Monitoring Patients With Sarcoma (EXOSARC)	Recruiting	Sarcoma	NCT03800121
Identification and Characterization of Predictive Factors of Onset of Bone Metastases in Cancer Patients	Recruiting	Bone metastases	NCT03895216
Predicting Prognosis and Recurrence of Thyroid Cancer Via New Biomarkers, Urinary Exosomal Thyroglobulin and Galectin-3	Recruiting	Thyroid cancer	NCT03488134
Anaplastic Thyroid Cancer and Follicular Thyroid Cancer-derived Exosomal Analysis Via Treatment of Lovastatin and Vildagliptin and Pilot Prognostic Study Via Urine Exosomal Biological Markers in Thyroid Cancer Patients	Active, not recruiting	Thyroid cancer	NCT02862470

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Akhil Srivastava: Conceptualization; funding acquisition; methodology; writing-original draft; writing-review and editing. **Narsireddy Amreddy:** Conceptualization; methodology; writing-original draft; writing-review and editing. **Vipul Pareek:** Conceptualization; formal analysis; methodology; writing-original draft; writing-review and editing. **Mahendran Chinnappan:** Conceptualization; methodology; writing-original draft; writing-review and editing. **Rebaz Ahmed:** Conceptualization; formal analysis; methodology; writing-original draft; writing-review and editing. **Meghna Mehta:** Conceptualization; data curation; methodology; writing-original draft; writing-review and editing. **Mohammad Razaq:** Conceptualization; data curation; investigation; writing-original draft; writing-review and editing. **Anupama Munshi:** Conceptualization; funding acquisition; methodology; supervision; writing-review and editing. **Rajagopal Ramesh:** Conceptualization; funding acquisition; methodology; supervision; writing-review and editing.

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