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Exosomes and extracellular vesicles: Rethinking the essential values in cancer biology

Akira Yokoi [a,b], Takahiro Ochiya [c,*]

[a] Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, Nagoya, Japan
[b] Institute for Advanced Research, Nagoya University, Nagoya, Japan
[c] Department of Molecular and Cellular Medicine, Tokyo Medical University, Tokyo, Japan

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ABSTRACT

Extracellular vesicles (EVs) such as exosomes are released by all living cells and contain diverse bioactive molecules, including nucleic acids, proteins, lipids, and metabolites. Accumulating evidence of EV-related functions has revealed that these tiny vesicles can mediate specific cell-to-cell communication. Within the tumor microenvironment, diverse cells are actively interacting with their surroundings via EVs facilitating tumor malignancy by regulating malignant cascades including angiogenesis, immune modulation, and metastasis. This review summarizes the recent studies of fundamental understandings of EVs from the aspect of EV heterogeneity and highlights the role of EVs in the various steps from oncogenic to metastatic processes. The recognition of EV subtypes is necessary to identify which pathways can be affected by EVs and which subtypes can be targeted in therapeutic approaches or liquid biopsies.

1. Introduction

Extracellular vesicles (EVs) are released by all living cells and are formed from bilayer lipid membranes. EVs consist of variety of subtypes, including exosomes, microvesicles (MVs), ectosomes, oncosomes, and apoptotic bodies; EV is understood to be a generic term that refers to secreted vesicles [1]. EVs are not just a simple lipid bilayer membrane structure; they are an important cargo carrier of various bioactive molecules [2], and these components of EVs can reflect the characteristics of the originating cells. EVs carry various cell-derived bioactive molecules, including proteins, nucleic acids, lipids, and metabolites, and circulate in extracellular spaces in biofluids such as blood, ascites, urine, and saliva.

A major historical turning point in this research field was the discovery of novel EV functions as the mediators of cell-to-cell interactions where EVs can deliver functional molecules to recipient cells resulting in the alteration of their physiological and pathological functions [3]. This breakthrough led to pioneering research in this new field. Intercellular communication between cells is an essential process for not only pathological situations but also normal homeostasis. Such crosstalk has been recognized as occurring via direct cell-to-cell contact or via secretion factors such as growth factors, cytokines, and chemokines [4–6]. However, over the past 10 years, a tremendous number of mechanisms involving cell-to-cell communication via EVs have been reported [2,7], and the characterization of EVs that contain various bioactive molecules has widened the involvement of scientists from various biological fields.

The potential for EV applications has been expanding quite rapidly, particularly in cancer-related fields [8,9]. In cancer biology, the role of EVs is now recognized as an essential factor for all processes in cancer progression. The tumor microenvironment (TME) comprises diverse cell types that actively interact with each other [10,11]. EV contents contribute to oncogenesis, metastatic disease, and resistance to chemotherapy [12,13]. Circulating EVs are also considered an essential clinical target for use as disease biomarkers as they carry tumor-specific molecular signatures such as neoantigen protein, RNA, and DNA, leading to the realization of liquid biopsies for cancer diagnosis and management [14,15]. Moreover, nano-technology-based approaches have also emerged to obtain high-quality EVs through easy and high-throughput methods [16]. EVs also have promising potential as therapeutics, as they can act as carriers to deliver therapeutic agents and contribute to other therapeutic approaches [17,18].

Recently, improved awareness of EV diversity has become an essential topic in this field [19] because the heterogeneity of EVs and presence of non-vesicular particles can be an obstacles that complicate

* Corresponding author at: Takahiro Ochiya Department of Molecular and Cellular Medicine, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjyuku-ku, Tokyo, 160-0023, Japan.
E-mail address: tochiya@tokyo-med.ac.jp (T. Ochiya).

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the understanding of the molecular composition and function of EVs [20]. Traditionally, condition- or origin-specific EVs have been analyzed to elucidate new pathways involved in cancer progression [21]. For instance, hypoxia-exposed cell-derived EVs had a high metastatic ability [22], and EVs from high metastatic cells generally have malignant phenotypes [12,23]. However, EV heterogeneity is more complicated than expected, and now most EV researchers regard understanding this issue as an important task for moving this field forward [8,16].

Numerous reports have revealed the essential value of EVs during the last decade, and substantial numbers of researchers from a variety of fields recognize these vesicles as providing targets to enable breakthroughs in various biological aspects. This review focused on updates of fundamental understandings of EVs from the aspect of EV heterogeneity and highlighted the role of EVs in cancer in the various steps from oncogenic to metastatic processes. We summarized the key roles of diverse EVs in TME and rethink their essential values in cancer biology to facilitate continuing and precise EV research. The recognition of EV subtypes is necessary to discuss further applications of how EV-dependent pathways can be targeted as therapeutic approaches or can be used for liquid biopsies.

2. Exosome and EV

Exosomes are one of the several subtypes from a diverse range of membrane vesicles and have unique characteristics including their specific components and biogenesis [24–26]. Historically, several scientists have described most small vesicles as exosomes, and it has been recognized as the useful way to indicate the specific research fields of EV analysis. However, an international scientific organization, the International Society for Extracellular Vesicles (ISEV), has developed standardized reporting for analysis of exosomes and other EVs and has recommended that the term “extracellular vesicle” should be used as a formal description for these vesicles; thus, there is now an increasing necessity to understand EV diversity [27]. Understanding the difference between exosomes and EVs is a good starting point and an important first step in starting EV research. In this section, we described the background of the emergence of exosomes and EVs.

2.1. Discovery of exosomes

In 1981, the term “exosomes” was first used to refer to membranous vesicles that are released from glioma cells [28]. However, the term “exosome complex” had also been used for an entirely different entity that was distinct from the current definition. In 1983, two papers reported at the almost same time that transferrin receptors associated with small vesicles were released from mammalian reticulocytes to the extracellular space during the maturation into erythrocytes [29–32]. Following these reports, Dr. Rose Johnston defined the name “exosome” for these EVs. However, these vesicles were long regarded as trash cargos of unnecessary components from cells [33–36].

2.2. The dawn of EV research

In 1996, important studies in EV research by Raposo et al. [37], and soon after by Zitvogel et al. [38] in 1998, reported that exosomes isolated from immune cells essentially function as activators of the immune system by maturing antigen-presenting cells (APCs) and activating T cells. After these reports, the value of EVs began to be recognized, and EVs were no longer considered as garbage cans [33].

A decade later, a milestone paper was published by Valadi et al. who discovered that both mRNA and microRNA were transferred between cells via small-EVs (S-EVs) [3]. S-EVs carrying RNAs from mast cells were transferred to other mammalian mast cells. After the transfer of mouse S-EVs containing RNAs to human mast cells, new proteins were detected in the recipient cells, suggesting that horizontally transferred mRNA can be translated in other cells. In other words, the EV components comprise bioactive molecules including nucleic acids. This discovery led to an explosive growth of EV research because this principle of transferring nucleic acid to other cells was a novel concept. EV-associated RNA can also influence recipient cells by altering RNA expression and the associated proteome, and these functions could be critical in intercellular communication to regulate immune responses [39] or many other types of pathophysiological responses [40,41]. This discovery opened a new field of research and significant advances have since been made in the identification of the biological significance of EVs as tools of intercellular communication.

2.3. Establishment of dedicated academic society

In response to an explosive global interest toward EV research, ISEV was established in 2011 for enhancing and organizing global EV research, focusing on exosomes, MVs, ectosomes, oncosomes, and other membrane-bound particles. The society has rapidly grown and now has over 1,500 members who interact internationally and help advance the study of EVs. Following the establishment of ISEV, this interest spread globally, resulting in the formation of various national societies including The American Society for Exosomes and Microvesicles (ASEMV), Society for Clinical Research and Translation of EVs Singapore (SOCRATES), Australia & New Zealand Society for EVs (ANSEV), UK Society for EVs (UKEV) as well as other societies based in Austria, Canada, France, Germany, Italy, Japan, Korea, the Netherlands, Spain, and Taiwan. In 2012, to expand research in the field, the ISEV launched a peer-reviewed open access journal, named the *Journal of EVs* (JEV) [39]. Besides research and review articles, the JEV periodically publishes ISEV opinion as position papers that are meant to promote standardization of EV research, and this acts as an essential resource to understand best practice in this field. In 2014, a set of minimal information guidelines for publication of EV studies was proposed in JEV, and this paper is known as “MISEV2014” [40]. This minimal information is regularly updated, and the most recent version is MISEV2018 [19,41]. This action promotes the standardization of EV study, and all EV scientists should know and follow these guidelines.

2.4. Nomenclature of extracellular vesicles

Recently, an expert consensus reported in a position paper that understanding the biogenesis and differences in the molecular contents of different-sized extracellular vesicles may also be relevant [19,42]. In this paper, it is clearly stated that the EV researcher should consider using operational terms for EV subtypes to refer to the following: (1) physical characteristics of EVs, such as size or density; (2) biochemical composition; and (3) descriptions of conditions or cell of origin. In regard to size-based definition, they defined S-EVs as < 200 nm diameter and large and/or medium EVs (m/l-EVs or L-EVs) as > 200 nm size. The terms “S-EV” and “m/lEV” are now recognized as a formal term to describe vesicles, and the ISEV recommended that scientists define specific EVs clearly and prominently at the beginning of each publication [43].

3. Biogenesis and EV diversity

At least two distinct major subtypes of EVs have been recognized based on their mechanism of biogenesis, endosome-origin “exosomes” and plasma membrane-derived MVs (or ectosomes/microparticles) [44,45]. The first step of EV biogenesis for both cases involves the inward/outward budding of the cell membrane (Fig. 1). Exosomes and MVs differ not only in their mechanism of formation but also in their components. Although it is difficult to isolate each group of EVs completely, understanding this difference is important to perform for further analysis.

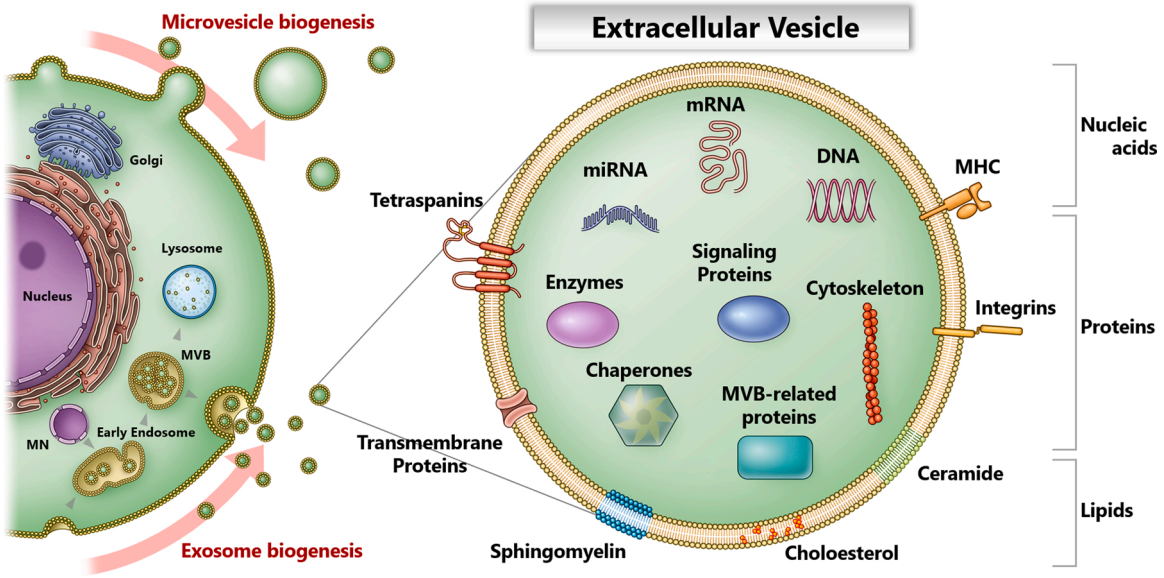

Fig. 1. Extracellular vesicle biogenesis and components. A. Microvesicle (MV) biogenesis comprises several steps, including plasma membrane reorganization, redistribution of phospholipids, outward repositioning of phosphatidylserine, disassembly of the cytoskeleton network, and actomyosin basal abscission. B. Exosome biogenesis starts inward of the plasma membrane to form early endosomes. Intraluminal vesicles (ILVs) are formed, and the endosomes mature to multivesicular bodies (MVBs). MVBs fuse with the plasma membrane to release ILVs into the extracellular space, where they are then referred to as exosomes. Alternatively, the MVBs can fuse with lysosomes, resulting in the degradation of ILVs. C. EVs can contain nucleic acids (DNA and/or RNA), membrane anchored-proteins, cytosolic proteins, and lipids; these contents can vary depending on the releasing cell types and their conditions.

3.1. Endosome-origin EV synthesis

Exosomes are a key type of EV that originate from the endosomal plasma membrane. The endosome undergoes a series of maturation steps and then forms multivesicular bodies (MVBs) that contain intraluminal vesicles (ILVs) [46]. Subsequently, MVBs traffic to and fuse with the plasma membrane, and consequently, they release their ILV contents into the extracellular space [47,48]. After this secretion process, ILVs are referred to as exosomes. This exosome synthesis pathway can be regulated by two parallel pathways: the endosomal sorting complex required for transport (ESCRT; ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III) dependent or independent pathways [49–54]. The process of fusing MVBs with the plasma membrane is regulated by Rab families, RAS-related proteins, or the membrane fusion soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex [55,56]. Recently, GTPases of the Ral family have been reported to control S-EV secretion via the activity of phospholipase D1 [57]. In addition, S-EVs from RalA/B-depleted cells have fewer malignant phenotypes such as a capacity for organotropism or promotion of metastasis. Fusion of MVBs with lysosomes results the degradation of the ILVs. Recently, it was demonstrated that the autophagosome may fuse with an MV in a pre-lysosomal step, resulting in a hybrid organelle termed an amphisome [58–61]. In general, EVs originating from endosomes tend to be positive for CD63 and of a smaller size than those derived from plasma membranes.

3.2. Plasma membrane-derived EV synthesis

Plasma membrane-derived EVs (pmEVs) consists of microvesicles, microparticles or ectosomes. PmEVs are formed by directly budding of the cell plasma membrane, and compared with exosome biogenesis, less is known about pmEV formation. PmEV release is MVB-independent and does not require exocytosis. A multistep process is followed: (1) reorganizing plasma membrane and phospholipid re-distribution; (2) phosphatidylserine repositioning; (3) disassembly of the cytoskeleton; and (4) actin–myosin basal abscission via the activation of ESCRT-I, MLCK and ADP ribosylation factor 6 [62–65]. It is recognized that plasma membrane-derived EVs reflect the surface proteins of parental cells and tend to be positive for CD9 and lager than endosome-origin EVs. Despite the distinct mechanism for biogenesis and membrane origin, both endosome-origin EVs and PmEVs can work similarly, and the crucial difference between them has not yet been elucidated [45].

3.3. EV diversity

As described above, EVs are diverse in terms of their size, content, morphology, and biological mechanisms [66–68], and single cells can continuously produce a diverse range of vesicles [69]. The heterogeneity of EV also reflects their cellular origin and functional impact on recipient cells. For instance, some S-EVs are positive for CD63, whereas others are negative (Fig. 2). Each S-EV has different functions in the immune system [47]. Moreover, the presence of small extracellular non-EV particles, such as exomeres, can act as obstacles to understanding true EV-related composition or functional properties [20,70,71]. Each subtype of EV may have distinct functions, and alternative methods are required to distinguish between these subpopulations. Therefore, it is critical that scientists involved in EV research understand the diverse and heterogenous nature of EVs.

3.4. EV components

EVs are carry a complex cargo in terms of specifically sorted nucleic acids, proteins, and lipids (Table 1), and this content is dependent on the status of the parental cell type or specific conditions, such as hypoxia or activation by some molecular factors [22,72,73]. In addition, the nature of the EV subtype is also a key factor in determining the molecular contents in EVs. For instance, larger EVs have more DNA, CD9, or Annexin A1 while smaller EVs carry CD63 and CD81. Each bioactive molecule has the potential to affect surrounding cells and to be considered as the target for therapeutic and biomarker applications.

3.4.1. Nucleic acids

EVs contain various forms of RNAs that can be transferred into recipient cells. RNAs packaged into EVs, undergo horizontal transfer

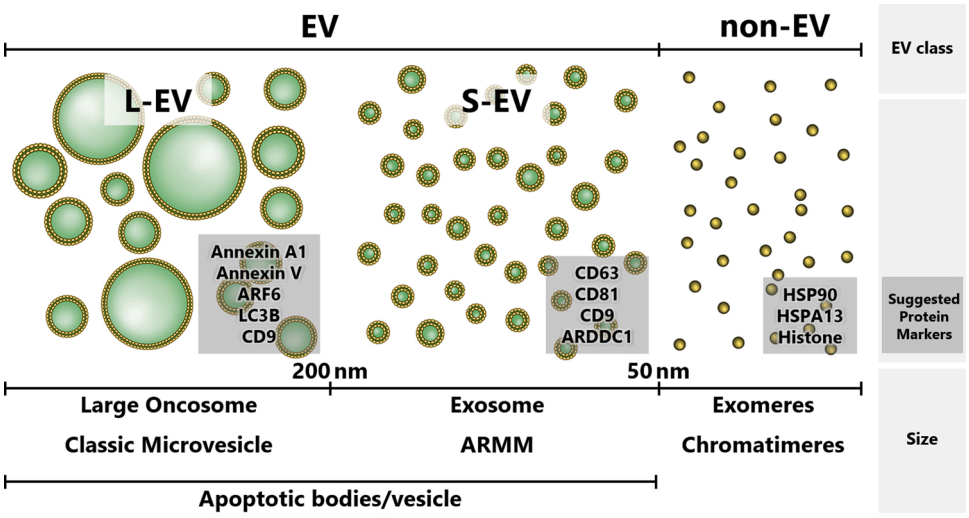

Fig. 2. Heterogeneity of extracellular vesicle. Diverse subtypes of EVs and non-EV populations. Currently, small-EVs (S-EV) are defined as being < 200 nm in diameter and large-EVs (L-EV) are over 200 nm.

Table 1
Summary of extracellular vesicle components.

Nucleic Acids	
DNA	Genomic DNA, Mutated DNA, Mitocondorial DNA
RNA	mRNA, miRNA, lncRNA, piRNA, circRNA,
Proteins	
Tetraspanins	CD9, CD63, CD81, CD82
Multivesicular body Biogenesis	Tsg101, Alix, Rab proteins
Heat shock proteins	HSP90, HSP70,
Growth factors and cytokine	TNF-α, VEGF, EGF, TNF Receptors, Transforming growth factor-β
Cell adhesion	Integrins, Intercellular Adhesion Molecule 1
Antigen presentation	MHC class I and II/peptide complexes
Signaling proteins	GTPase HRas, Ras-related protein, Src, RhoA
Cytoskeleton components	Actins, Cofilin-1, Moesin, Myosin, Tubulins, Vimentin
Transcription and protein synthesis	Histone, Ribosomal proteins, Ubiquitin
Metabolic enzymes	Fatty acid synthase, Phosphoglycerate, ATPase, Aldehyde reductase
Death receptors	FasL, TNF-related apoptosis inducing ligand
Iron transport	Transferrin receptor
Lipids	
Ceramides (nSMase2)	
Cholesterol	
Leukotriene (LTA4, LTB4, LTC4)	
Cyclooxygenases (COX-1, COX-2), Prostaglandins	
BMP (Bisphosphate)	
Phosphatidylserine	
Sphingomyelin	

between cells, resulting in transient or persistent phenotypic changes of recipient cells [74]. According to RNA sequencing analysis, microRNAs (miRNAs) were the most abundant RNA form in human plasma EVs and account for approximately 40 % of all sequencing reads [75]. EV-related miRNAs have been extensively studied and they are understood to have numerous functions in all physiological and biological cascades [12,76,77]. Other types of RNA such as mRNA, long noncoding RNA (lncRNAs), piwi-interacting RNA (piRNA), and circular RNA (circRNAs) are also found in EVs and their functions have been reported. Functioning lncRNAs and circRNAs have recently been reported from EVs and can affect a variety of biological processes, including cancer progression [78]. The existence of DNA has been also reported [79] and especially in S-EVs, although this is still under discussion [20]. Recently, the mechanism of how DNAs are loaded into S-EVs, especially in MVB-dependent exosomes, was reported and the nuclear contents were shown to be shuffled to the exosome biogenesis pathway via collapsing micronuclei [80]. An alternative theory was recently proposed whereby active secretion of DNAs may occur through an amphisome-dependent mechanism [58–61].

3.4.2. *Proteins*

EVs carry many types of proteins, the nature of which depends on the function of the associated cell type [8]. EV proteomes of various origins include tetraspanins (CD9, CD63, CD81, and CD82), MVB-related proteins (TSG101, ALIX, and Rab proteins), heat shock proteins (HSP90 and HSP70), growth factors and cytokines (TNF-α, VEGF, EGF, TNF receptors, and transforming growth factor-β (TGF-β)), cell adhesion-related proteins (integrins and intercellular adhesion molecule 1), antigen presentation-related proteins (major histocompatibility complex (MHC) class I and II/peptide complexes), signaling proteins (GTPase HRas, Ras-related protein, Src, and RhoA), cytoskeleton components (actins, cofilin-1, moesin, myosin, tubulins, and vimentin), transcription and protein synthesis-related proteins (histone, ribosomal proteins, and ubiquitin), metabolic enzymes (fatty acid synthase, phosphoglycerate, ATPase, and aldehyde reductase), death receptors (FasL and TNF-related apoptosis-inducing ligand), and iron transport proteins (transferrin receptor). These molecules can have functional effects on their recipient cells, and therefore the localization of proteins in EVs is an important factor in the interactions with recipient cells [81,82]. Furthermore, membrane proteins can be used as disease biomarkers because EVs can carry unique proteins that reflect their specific patient conditions. However, the isolation methods can easily affect the characteristics of EV proteins because a range of non-EV-related proteins, such as albumins or immunoglobulins, can contaminate purified EV samples [83]. The issue of contamination is particular critical with human biofluids, and therefore this aspect of obtaining pure EVs has been keenly discussed. However, a consensus has yet to be reached on the most effective way to purify such EVs [14,84].

3.4.3. *Lipids*

As the EV surface comprises components of the plasma membranes, this contains various types of lipids, including ceramide, cholesterol, sphingomyelin, and phosphatidylserine as well as saturated fatty acids [48]. EVs may have several functional lipolytic enzymes that produce various bioactive lipids that can be internalized into surrounding cells [85]. Ceramide is one of the most abundant lipids and is important for EV formation. Lipid-enriched EVs may stimulate cell signaling pathways in cancer phenotypes [86]. Glioblastoma-derived S-EVs containing

α-galactosylceramide enhanced T-cell activation by dendritic cells (DCs) [87], which is a promising strategy as part of DC-based immunotherapy. Phosphatidylserine lipids can be used as cancer detection biomarkers, and lactosylceramide in urine has also been reported as a prostate cancer biomarker [88,89]. Furthermore, the function of lipids for EV uptake was recently reported [90]. In glioma models, S-EVs induced lipid droplet accumulation in hypoxic glioma cells and their uptake depended on high expression of increased heparan sulfate proteoglycan endocytosis and the lipid raft-related pathway. However, compared with nucleic acids and proteins, EV-related lipid functions are not as well understood.

4. EV in cancer biology

EVs have been widely tested in models of cancer cell-related in vitro/in vivo systems to confirm their use as newly emerged biological tools. From the beginning of EV research, EV-related pathways were investigated using cancer cells [3], and to this date, there is abundant evidence supporting the concept that EVs secreted from both tumors and their surrounding cells have essential functions in cancer biology [6,8,91–93]. Tumor-secreted EVs are critical mediators of intercellular communication in local and distant microenvironments (Fig. 3). However, to interpret the data from literature, we have to be especially careful to assess how the EVs under investigation have been characterized in the study. According to MISEV2018, the ISEV requires the a detailed description of EV characteristics, including their size, quantity, and molecular composition as well as the details of any marker proteins and how the EVs were imaged; the ISEV also recommends that multiple methods are used for isolation and analysis to ensure reproducibility [19]. Failing to provide such details of the characterization methods and results can lead to serious issues and affect the reproducibility of the work. This next section summarizes pathophysiological functions of EVs involved in each cancer progression process.

4.1. EVs in tumor microenvironment

Currently, EVs are considered the key components in the tumor microenvironment (TME) associated with cancer cells [94]. When a tumor forms at the primary site, the tumor cells require active communication with neighboring cells and their local microenvironment. The TME comprises cellular and non-cellular factors contributed by the tumor and surrounding cells. EVs are now understood to be key players in cell-to-cell mediation. The main composition in the TME includes extracellular matrix (ECM), fibroblasts/cancer-associated fibroblasts (CAFs), inflammatory-related immune cells, and tumor-associated vasculature including endothelial cells [95]. In the TME, the events of critical importance for cancer cell progression continuously occur, and tumors gradually obtain malignant phenotypes. During this window, EVs are believed to critically participate in multiple steps from oncogenesis to the early steps involved in metastasis.

4.1.1. Oncogenesis

Oncogenesis is the initial stage where healthy cells become abnormally transformed into cancer cells, and this comprises a multistep process. Cancer cell-derived EVs have been implicated in determining the tumorigenic potential of normal cells [96]. These functions are not limited to S-EVs [97,98] but are also present in L-EVs [99]. S-EV-related miRNAs, classically called exosomal miRNAs, may also contribute to tumorigenesis, and only cancer-derived S-EVs can mediate cell-independent miRNA production. S-EVs with RNA-induced silencing complex-associated miRNAs have been shown to induce tumor formation from healthy mouse mammary cells [97]. In contrast, EVs from noncancerous neighboring epithelial cells can suppress cancer initiation [100], which may be related to the concept of cell competition [101].

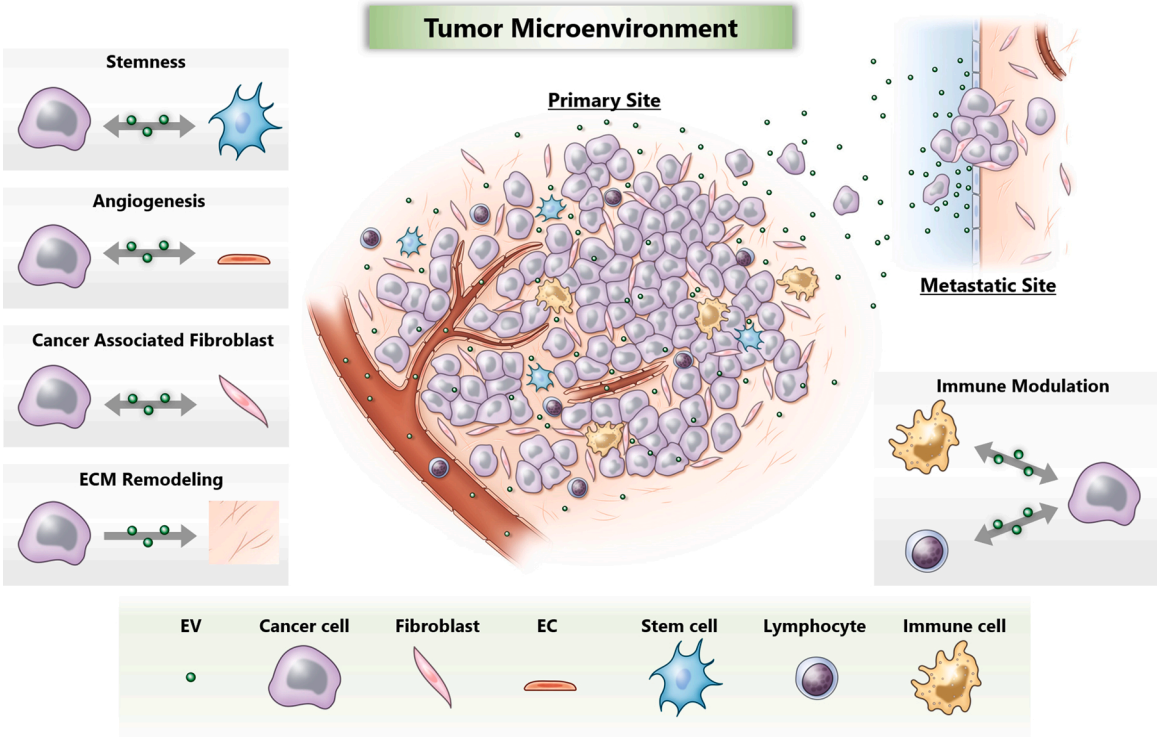

Fig. 3. Role of extracellular vesicles in tumor microenvironment. In the tumor microenvironment, cancer cells are actively communicating with neighboring cells such as fibroblasts, endothelial cells, and various immune cells via EVs. The EVs can also affect the extracellular matrix. The interaction of cells with EVs is bidirectional, resulting in many events, including oncogenesis, angiogenesis, immune modulation, extracellular matrix (ECM) remodeling, epithelial-to-mesenchymal transition (EMT), and cancer-associated fibroblasts (CAF) transformation. These events contribute to cancer cells into the circulation and their spread to distant sites. Metastasis can occur by hematogenous, lymphatic, or direct dissemination via biofluids such as ascites.

Recently, prostate cancer cell-derived S-EVs enriched in miR-424 were proposed to induce stemness and tumorigenesis in normal epithelial cells [102]. During cancer initiation, there is a conflict between newly transformed cells and surrounding epithelial cells, and this may be due to transferring tumor-suppressive miRNAs via S-EVs.

4.1.2. Angiogenesis

EVs have been recognized as strong contributing factors for angiogenesis. Angiogenesis is a dynamic process involving angiogenic factors, ECM components, and endothelial cells (ECs) [103]. The interaction between cancer cells and ECs via EVs has been extensively investigated. Several groups showed that cancer cell-derived S-EVs promote angiogenesis in vitro and in vivo systems [104–106]. Hypoxia can also alter the characteristics of EVs, and these EVs can be a crucial factor in this condition. For instance, S-EVs from hypoxic glioblastoma cells induced proangiogenic programming of ECs and cancer cell proliferation [107]. The vascular permeability and extravasation of cancer cells by cancer-derived EVs are an essential step for cancer progression [108]. For example, cancer-derived S-EVs are incorporated into ECs, and their contents regulate EC transcription factors that modulate vascular permeability [109]. These phenomena are also important for distant metastasis, as described in the following section.

4.1.3. Epithelial-to-mesenchymal transition phenotype

For cancer cells, the epithelial-to-mesenchymal transition (EMT) is the key process of invasion, chemoresistance, and metastasis [110]. This involves both intracellular signaling pathways and extracellular modulators in the TME that include EVs that induce cells to undergo EMT [111]. HRAS-overexpressing cells promote the packaging of mesenchymal markers such as vimentin and MMPs into S-EVs, potentially inducing EMT in recipient cells [112]. S-EVs isolated from the metastatic breast cancer cell line MDA-MB-231 stimulated with linoleic acid induced an EMT-like process in epithelial MCF10A cells [113]. EVs from mesenchymal stem cells (MSCs) have been shown to stimulate EMT induction and promotion. The research field of MSC-derived EVs is quite broad and, regarding the function of the EVs, it is important to understand the source of MSCs, such as whether they are from bone marrow, umbilical/placental, or adipose tissues [114,115]. For example, S-EVs derived from human umbilical cord MSCs can promote proliferation, migration, and invasion through the activation of the ERK signaling pathway in breast cancer cells, and EVs from adipose-derived mesenchymal stem cells can promote migration through the Wnt-dependent pathway [116,117]. Elucidating the role of EVs in the regulation of the EMT can potentially be useful for biomarker applications or therapeutic targets [118].

4.1.4. ECM remodeling

ECM remodeling is commonly thought to initiate the invasive phenotype of tumors. S-EVs carry active proteases capable of ECM degradation such as collagens, LNs, and fibronectin, and those S-EVs contribute to ECM remodeling by selectively and directly binding to the ECM-binding motif present on surface adhesion proteins [119]. Rab27b-mediated exocytic release of HSP90 via S-EVs from metastatic cancer cells was shown to activate MMP2, resulting in the degradation of ECM components, release of growth factors, and promotion of cancer cell invasion [120]. This process is important for cancer cells to create favorable conditions in the TME and generate a metastatic niche.

4.1.5. CAFs

Cancer cells induce aerobic glycolysis in neighboring stromal cells such as fibroblasts, and these fibroblasts are defined as CAFs [121]. CAFs support important features of cancer progression, including tumorigenesis by stimulating angiogenesis, cancer cell proliferation, and invasion [122]. CAFs are essential cells in TME and communicate with surrounding cells through various mechanisms. The bidirectional interaction between cancer cells and CAFs is quite significant; for instance, EVs from cancer cells activate fibroblasts to transform to CAFs and the EVs from CAFs can then affect the tumor phenotype [123]. The S-EVs from CAFs promote cancer cell progression and include delivery of astrocyte-derived miR-19a to cancer cells, resulting in PTEN suppression and contributes to metastasis [124]. The CAF-S-EVs also stimulate the migration of cancer cells by stimulating Wnt-PCP autocrine signaling [125]. In the ovarian cancer model, the S-EVs of CAFs suppresses apoptosis and confer chemoresistance by direct binding to the novel target, APAF1 [126]. The S-EVs derived from highly metastatic cancer cells reprogrammed fibroblasts into CAFs compared with those derived from non-metastatic cancer cells [127,128]. Recently, the role of S-EVs related miR-375 toward CAF induction was reported by using Merkel cell carcinoma, which is a highly malignant skin cancer [129]. mir-375 does not affect cell malignancy itself but the transfer to fibroblast resulting the induction of CXCL2, α-SMA and IL-1β. In summary, CAFs play a major role in the maintenance of TME by releasing and receiving EVs and are involved in various signal transduction pathways [125].

4.1.6. Immunological involvement

Immune cells are also one of the major contributors in the TME, and EVs maintain this microenvironment by immune system modulation. Cancer cell-derived EVs involved in immunosuppression have important functions as they confer advantages to cancer cells to escape attack from immune cells [130]. EVs suppress natural killer (NK) cell and T-cell activity to enhance immune evasion [131–133]. Yen et al. reported that the S-EVs from cancer cells promote the expansion of regulatory T cells by TGF-β1 in S-EVs [134]. Cancer-derived S-EVs have also been reported to promote the activity of myeloid-derived suppressor cells assisting cancer cell dissemination [135]. Lung cancer-derived EVs transferred into epithelial cells result in the activation of toll-like receptor 3 (TLR3) in lung epithelial cells and recruitment of neutrophils leading to the formation of a premetastatic niche (PMN) in the lung [136]. Hypoxic conditions affect the function of EVs, and cancer-derived L-EVs from hypoxic conditions are enriched in miR-23a, resulting in the blockade of NK cell function [137].

The function of S-ECs from DCs was described in a milestone study [138]. These EVs interact with T cells with the aid of APCs and can trigger immune responses. DCs secrete antigen-presenting S-EVs, which express functional MHC class I and class II molecules and T-cell costimulatory molecules. The S-EVs prime cytotoxic T lymphocytes and suppress tumor growth in vivo [38]. The therapeutic potential of DC-derived S-EVs has been well investigated, and engineered EVs from DCs have been shown to control the modulation of immune cells and influence anticancer immune responses [139].

Recently, the successful application of the immune checkpoint inhibitors, including anti-PD-1/PD-L1 and anti-CTLA-4 has established immunotherapy as an irreplaceable and viable treatment option for patients with advanced cancers [140]. The existence of immune checkpoint molecules on EVs was reported a while ago [141], but the evidence that these molecules on EVs are actively involved in these pathways has only recently been accumulating [142,143]. Interferon-gamma (IFN-γ) upregulated PD-L1 expression on S-EVs derived from glioblastoma, and S-EVs induced PD-L1-dependent inhibition of T-cell function, resulting in suppression of antitumor immunity [133]. In chronic lymphocytic leukemia, noncoding Y RNA hY4 in S-EVs stimulated monocytes to secrete cytokines, including CCL2, CCL4, and IL-6, and the stimulated PD-L1-mediated immune escape [144]. S-EVs from metastatic melanomas also carried PD-L1 on their surface, and stimulation with IFN-γ increased the amount of PD-L1 on these vesicles, which then suppressed the function of CD8 T cells and facilitated tumor growth [145]. Moreover, PD-L1 carrying S-EVs also suppressed T-cell activity in the draining lymph node, and tumor cells without these EVs induced systemic antitumor immunity and memory [146]. In a hepatocellular carcinoma model, ER stress promoted S-EV secretion to stimulate PD-L1 activation in macrophages and then suppressed T-cell function via the miR-23a-PTEN-AKT regulated pathway [147].

4.2. Distant metastasis

Metastasis is a unique phenomenon that involves intense cell–cell communication in the TME. A complex multistep process is required for successful growth of metastatic lesions that includes steps such as the invasion of surroundings, survival in blood vessels, escape from immune attack, and attachment to and colonization of host organs [148]. EVs have important roles in each of these steps, and the functions of EV-related metastasis has been well studied. As EVs are secreted factors from cancer cells, they can affect both proximal and distant sites and thereby influence metastatic processes [149,150].

A PMN is defined by the creation of an environment distant from the primary tumor that is suitable for the colonization of incoming circulating tumor cells, and each tumor type tends to metastasize to specific organs, suggesting that the microenvironment plays a role in dictating metastatic invasion [151,152]. S-EVs derived from CD44-variant isoform-positive pancreatic cancer cells contribute to the formation of PMNs in the lymph nodes and lungs [153]. L-EVs derived from CD105-positive renal cell carcinoma created PMNs by enhancing the expression of VEGF and MMP2 in lung ECs [154]. S-EVs derived from highly metastatic mouse melanoma cells distributed to the lung and bone marrow forming PMNs to enable colonization by cancer cells [155]. Vascular leakiness was a key phenotype in these reports and is considered a hallmark of PMN formation [156,157]. Following these studies, Hoshino et al. elucidated the mechanism of this metastatic organotropism via EVs. These authors revealed the functions of diverse integrins on S-EVs and demonstrated that specific integrin carrying EVs enable organotropism and the formation of PMNs at specific sites [157].

The blood–brain barrier (BBB) is a strict construction that protects the central nervous system and is also considered the main obstacle for the creation of metastatic lesions at the brain because cancer cells have to recognize and bind to components of the vascular endothelial membrane to initiate their extravasation and invasion [158,159]. Recently, the potential of EVs that contribute to this metastasis has been reported. S-EVs containing miR-181c are transferred from metastatic brain cancer cells to brain ECs, resulting in the destruction of tight junction proteins of the BBB, including claudin-5, occludin, and tight junction protein 1 (ZO-1), which bind to actin, the primary cytoskeletal protein. These S-EVs promote the destruction of the BBB through the delocalization of actin fibers via the downregulation of PDPK1 by miR-181c, which causes the subsequent degradation of phosphorylated cofilin and the severing of actin filaments [160]. miR-105 in breast cancer S-EVs also suppresses ZO-1 expression in ECs, resulting in the loss of cell–cell adhesion, thereby promoting metastasis [161].

EVs also contribute to the non-hematogenous metastasis such as peritoneal dissemination that frequently occurs in ovarian and gastrointestinal cancer [162]. For instance, S-EVs from highly metastatic ovarian cancer cells promote metastasis in vivo and were enriched with MMP1 [23]; mRNAs are horizontally transferred to the peritoneal mesothelium, causing apoptosis of the peritoneal mesothelial cells. This destructive phenotype is considered as the formation of a PMN and enables cancer cells to generate metastatic lesions. Following this report, various other studies also supported this concept of EV-related peritoneal metastasis [163–165].

Cancer patients can develop recurrent metastatic tumors long after their first treatment. This phenomenon can be explained by cancer cell dormancy, where there is a growth arrest of cancer cells in tissues such as the bone marrow [166]. Growing evidence highlights the role of EVs in this process [167–169]. For instance, S-EVs released from bone marrow MSCs promotes breast cancer cell dormancy by transferring miR-23b, which regulates the gene, *MARCKS*, that encodes the cell division and motility-related protein myristoylated alanine-rich C-kinase substrate [169].

5. EVs as a cancer biomarker

Studies of EV cell biology are not only important to reveal new cell biological pathways but are also critical to open new avenues for clinical use of EVs as disease biomarkers [170]. EVs have maximum potential as the target of liquid biopsies as they can be detected in almost all types of body fluids such as blood, urine, saliva, breast milk, and ascites [171]. EVs can then be used as biomarkers to diagnose diseases in non-invasive or minimally invasive ways [172]. As explained above, each EV has powerful and specific functions that can be directly recognized as potential biomarkers. EVs can be isolated from all biofluids for this purpose and contain a tremendous number of molecules that can be used as target biomarkers. To discover reliable biomarkers for detecting cancer at early stage, an ideal biomarker should be highly specific for a primary tumor type and be reproducibly detectable at premetastatic stages using non-invasive techniques, and several studies have already attempted these challenges [173].

5.1. EV-related molecular targets for cancer biomarkers

As described in prior sections, EVs carry various molecules such as proteins, nucleic acids, or lipids. Each molecule can be considered as a potential biomarker for a disease because they can reflect patient status. Among those molecules, proteins and nucleic acids have been well investigated during the past decade for use biomarkers.

5.1.1. EV-protein biomarkers

There has been intensive research on identifying disease-specific EV proteins. Numerous EV-protein targets have been reported, and one classic target is the epithelial cell adhesion molecule (EpCAM) [174]. Levels of EpCAM-positive S-EVs were reported to increase during ovarian cancer progression and to be significantly higher in patients with ovarian cancer than in women with benign ovarian disease or in healthy controls [175]. However, EpCAM is not specific for ovarian cancer and this method has not progressed. In melanoma, S-EVs from the blood of melanoma patients are enriched in caveolin-1, S100B, and Melan-A compared with those of healthy controls, enabling caveolin-1-positive S-EVs to act as potential melanoma biomarkers [176–178]. In patients with pancreatic cancer, the level of MIF in S-EVs may serve as a prognostic marker for liver metastasis [179]. Regarding immune therapy, PD-L1 on S-EVs can serve as a promising biomarker for differentiating clinical responders from non-responders [145]. From the aspect of organotropism, the α6β4 and α6β1 integrins on S-EVs are associated with lung metastasis, whereas the αvβ5 and αvβ3 integrins on S-EVs are associated with liver and brain metastasis, respectively [157]. Thus, these EVs can be predictive biomarkers for the occurrence of metastasis. Furthermore, large-scale proteomics data for human biofluids, including plasma/serum, bone marrow plasma, bile duct fluid, and lymphatic fluid was recently reported to support the feasibility of EV-related proteins for cancer detection [180]. Over one hundred samples from cancer patients were compared with those of normal controls and several proteins were specifically identified as cancer biomarkers. However, looking at each cancer type, the sample size was not large enough to confirm their use as biomarkers and further validation seems to be required.

5.1.2. Nucleic acid EV biomarkers

miRNAs within circulating EVs have also been well investigated and their diagnostic and prognostic potential revealed in many cancer types [181–184]. S-EV-related miRNAs in urine can be targeted, especially in urogenital cancers such as prostate cancer [181], and can also reflect the clinical backgrounds of metastasis or recurrence [23,160,185,186]. The combination of multiple EV-miRNAs enhances diagnostic performance, and these miRNA signatures are longitudinally emerging in association with patient status [187,188]. Recent advances in this field may involve the application of machine/deep learning to establish the best

algorithms for signature discovery [189]. After performing global analysis such as sequencing or microarray analysis, a bioinformatics approach can help to create accurate predictive models [190]. For example, in ovarian cancer, whole serum miRNA profiles were obtained by miRNA microarray analyses from 4046 serum samples and then narrowed down to targets based on whether these were S-EV-dependent miRNAs or not [191]. As a result of machine learning analyses, a 10 miRNA combination could be used to differentiate cancer patients from non-cancer patients with extremely high accuracy.

Other genetic material in EVs also has potential as biomarkers. Tumor-specific mRNA isolated from L-EVs from glioblastoma patients reflects the mutational status of EGFRvIII [192,193]. Other RNAs such as lncRNA, circRNA, or piRNA can also be considered as targets for biomarkers, but EVs carrying DNAs are attracting a great deal of recent attention because these could act as less-invasive biomarkers for cancer for direct analysis of the tumor genome [194]. In comparison with cell-free DNA, EV-related DNA remains poorly understood [195]. According to previous reports, S-EV carrying double-stranded DNA reflects the oncogenic status of the parental cancer cell [196–199], demonstrating the utility of EV-related DNA as a feasible biomarker in the clinical setting. S-EVs carrying DNA were more abundant in melanoma patients in advanced stages compared with those in early stages [197]. Furthermore, by using plasma derived from patients with metastatic melanoma, assessment of the S-EV subpopulations could improve the detection of mutated DNA compared with that of cell-free tumor DNA [200]. This is a fascinating result that illustrates the importance of investigating EV-DNA in clinical oncology. L-EVs are also a promising target for investigating EV-DNA. Importantly, L-EVs can also contain single-stranded DNA carrying the specific genomic alternations of primary tumor, such as oncogene MYC amplifications [79], and some L-EVs have a higher amount of DNA than that in S-EVs [201]. Recently, it was revealed that the DNAs in S-EV from metastatic ovarian cancer patient-derived ascites clearly reflect the copy number variation status of the primary tumor [80], indicating that specific genetic alterations can also be targeted.

5.2. Technology-based approaches for cancer biomarkers

The isolation of EVs is a hot topic in EV research because a gold standard method remains undefined. Regarding clinical samples, it remains challenging to isolate target EVs with high purity that can then be considered for clinical application, where accurate performance and high-throughput methods are prioritized [19,27]. To overcome some of the limitations of EV isolation from human samples, the use of technology-based innovative devices is an attractive option [16], and these novel methodologies have various advantages, including high specificity, small testing volume, easy and simple steps, and high throughput. In 2014, Im et al. developed the nanoplasmonic exosome assay, which is based on the concept of surface-plasmon resonance, enabling direct label-free and high-throughput analysis of proteins in EVs [202]. The assay focused on the detection of EpCAM and CD24 in ovarian cancer patient samples. In the same year, Yoshioka et al. reported an amplified, solution-based luminescent proximity homogeneous assay for detecting circulating EVs [203]. This method also does not need any purification steps before analysis. This procedure enables analyses of EVs from urine using a ZnO nanowire-based methodology to capture urinary EVs and to simultaneously extract their miRNAs [204]. These microfluidic chip-based approaches have been widely investigated and evidence for their feasibility in clinical applications is accumulating [205–208]. However, to interpret the data, the EVs purified by a device should be fully characterized. The quality of EVs should always be validated, and readers should pay careful attention to this aspect of a study.

6. EVs as cancer therapeutics

This section highlights the fascinating potential for EVs as therapeutic tools for cancer treatment. EVs are naturally taken up by target cells and can be used for transferring therapeutic agents and are therefore being considered as highly attractive candidates for potential tumor-targeted vehicles in cancer therapy [209]. In contrast to other nanomaterials, such as liposomes, metals, or polymers, injected EVs can efficiently enter target cells and deliver functional agents with minimal immune clearance [210–212]. For these reasons, engineered EVs have been considered to help realize efficient drug delivery systems. For example, macrophage-derived S-EVs loaded with paclitaxel significantly increased cancer cell uptake of the drug, and S-EV-mediated chemotherapy delivery enhanced drug effects [213]. Furthermore, EV-based delivery platforms have great potential to reduce side effects because the complex comprises completely natural molecules [214]. Nucleic acids can also be used as potential agents that show anticancer effects. Engineered S-EVs containing a miR-21 sponge downregulated miR-21 expression in target cancer cells and this resulted in the upregulation of target genes, such as PDCD4 and RECK [215]. In a triple-negative breast cancer model, the S-EV surface derived from macrophages was coated with polylactic-co-glycolic acid and then further engineered with a specific peptide for targeting the mesenchymal–epithelial transition factor; these modified S-EVs increased the efficacy of cellular uptake and the antitumor capacity of doxorubicin [216].

EVs themselves can also be used as therapeutic tools. For instance, EVs from mesenchymal cells are not toxic in in vivo experiments [217,218], and MSC-derived S-EVs have been considered to be therapeutic by themselves [219] because of their low immunogenicity and immunosuppressive activity. This has been clinically tested in the treatment of a patient with graft versus host disease, and these S-EV injections were well tolerated without significant side effects, resulting in a favorable patient response [220]. In contrast, cancer-derived S-EVs can stimulate antitumor immune responses and generate prophylactic immunity. This concept was tested as an antitumor vaccine, and the effect of DC-derived S-EVs was assessed [221,222]. Preclinical assessment of MSC-derived S-EVs for therapeutic use covers numerous categories, including neurological, cardiovascular, immunological, and kidney diseases [115]. Among these, the cancer-targeted therapeutic strategy remains an minor category, but preclinical studies in this area have been increasing [223,224]. Various methods have been reported to utilize MSC-derived S-EVs, including engineering or modifying the EV structure and targeting the cargo for drug delivery.

Alternative therapeutic strategies are inhibition of EV release from cancer cells [225] or to eliminate the EVs themselves [226,227], but the effects of these options have not yet been verified in a clinical setting. Additionally, several clinical trials have already produced important results [228]. A phase II trial of IFN-γ-DC-derived S-EVs boosted antitumor immunity via NK cells in non-small cell lung cancer patients [229]. Multiple clinical trials are now ongoing, and these therapeutic concepts are no longer unique [230]. Regarding clinical applications, the issue of large-scale production and storage are still considered as challenging and remain a topic of discussion in the field.

7. Current discussions and perspectives

Following the recognition of exosomes, the field of EV research has dramatically changed over recent decades and continues to rapidly expand. The concept that secretary messengers from cells contain a complex of bioactive molecules is now globally accepted, and EVs have gathered much attention from many different scientific specialists. The ISEV has worked on essential guidelines to help standardize the understanding and experimental handling of EVs. The need for precise and accurate characterization of EVs is still very much required, and the key issues will be in understanding the heterogeneity of EVs. In the cancer biology field, the bidirectional transfer of EV-related molecules between

tumor cells and the microenvironment has been much studied, and promising functions have been identified. However, very few reports mentioned the diversity of EVs. For instance, if the promising phenotypes of EVs are reported, the characteristic differences between S-EV and L-EV, or MBV-dependent and MBV-independent S-EV, have not been described in as much detail. The biodistribution of diverse EVs also remains poorly understood. This situation might be attributable to the difficult challenges of EV handling such as standardizing methods for EV isolation, quantification, and analyses. Detection sensitivity and specificity are still considered as major challenges for the characterization of each subpopulation and individual vesicle composition. Technological advances may help to resolve these issues, but the first step should recognize this limitation. Hence, assessments for EV heterogeneity should be investigated and novel insights will be revealed in the near future. There is no longer any doubt that EVs hold great potential to address cell biology questions, and it is also critical to develop new opportunities for their clinical use as biomarkers and therapeutics. To realize the future of using EVs in clinics and improve outcomes for cancer patients, further studies to continue developing fundamental understanding are necessary, and the current stream of EV research is on track to achieve this.

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Declaration of Competing Interest

There is no COI to disclose.

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