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NON-THEMATIC REVIEW

The roles and implications of exosomes in sarcoma

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Abstract Better diagnostic biomarkers and therapeutic options are still necessary for patients with sarcomas due to the current limitations of diagnosis and treatment. Exosomes are small extracellular membrane vesicles that are released by various cells and are found in most body fluids. Tumor-derived exosomes have been proven to mediate tumorigenesis, intercellular communication, microenvironment modulation, and metastasis in different cancers, including in sarcomas. Recently, exosomes have been considered as potential biomarkers for sarcoma diagnosis and prognosis, and as possible targets for sarcoma therapy. Moreover, due to their specific cell tropism and bioavailability, exosomes can also be engineered as vehicles for drug delivery. In this review, we discuss recent advances in the roles of tumor-derived exosomes in sarcoma and their potential clinical applications.

Keywords Exosome · Sarcoma · Biomarker · Targeted cancer therapy

1 Introduction

Bone and soft-tissue sarcomas are a large and heterogeneous group of malignant primary neoplasms derived from mesenchymal origin [1]. Surgery, radiation therapy, and chemotherapy are the standard treatments for sarcomas. The application of chemotherapy in addition to surgery has significantly improved the survival rate and quality of life of patients with certain types of sarcomas, including osteosarcoma, Ewing sarcoma, and rhabdomyosarcoma [2, 3]. However, progress has slowed over the past 30 years, and efforts to improve outcomes with intensifying regimens or adding novel agents have brought disappointing results. Specifically, for sarcoma patients with metastatic disease at diagnosis and for those with relapsed disease, outcomes are significantly poorer with the overall survival rate at less than 20 % [4]. Furthermore, current chemotherapeutic agents are generally not effective in some extracellular matrix (ECM)-rich types of sarcomas, including in chondrosarcoma and chordoma [5, 6]. Therefore, the development of novel therapeutic strategies is critical for this patient population. Recent discoveries of tumor-derived exosomes in sarcomas may open a new front in the battle against these malignancies.

Exosomes were initially described as a mechanism for the cellular release of waste and toxins [7]. Since then, substantial data have illustrated exosomes as important mediators of extracellular signaling *via* the membrane-protected transferring of cellular material. Although exosomes can be released by both normal and malignant cells, tumor cells usually secrete significantly higher amounts of exosomes [8–10]. Exosomes have now been recognized to hold important roles in tumorigenesis, apoptosis, immune response, and chemotherapeutic resistance [11–15].

Recently, exosomes have been highly considered as useful non-invasive biomarkers for cancer diagnosis and prognosis and a possible target for cancer therapy. Although exosomes have been intensively studied in different cancers, little is known about their existence and possible role in sarcomas. In this review, we will summarize the most recent studies on the roles of tumor-derived exosomes in different sarcomas and

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discuss the potential applications of exosomes in the treatment of sarcoma.

2 Mechanisms of biogenesis, secretion, and uptake of exosomes

Exosomes are one sub-type of extracellular vesicles that include several heterogeneous groups of membrane-limited vesicles loaded with various proteins, lipids, and nucleic acids. Extracellular vesicles can be classified based on their cellular origin, biological function, or biogenesis pathways (Table 1). Within the past decade, exosomes have emerged as the most important extracellular vesicles to mediate intercellular communication and regulate a diverse range of biological signals and processes. Exosomes are extracellular 40 to 100 nm vesicles secreted by a wide range of mammalian cell types. Exosomes typically consist of a lipid bilayer membrane surrounding a small cytosol and are devoid of cellular organelles (Table 1). The raft-associated lipids of exosomal membrane mainly consist of cholesterol, sphingomyelin, ceramides, and phosphatidylcholine [29]. Exosomes contain various molecular constituents of their parental cell, including proteins (CD9, CD63, and CD81), DNAs (single- and double-stranded), and RNAs (messenger RNA (mRNA), microRNA (miRNA), and long non-coding RNA (lncRNA)) (Fig. 1) [30–34]. The contents of exosomes are determined by diverse conditions and different donor cell types and can reflect different gene expression profiles due to the selective sorting of exosomes from the donor cells.

The molecular mechanism of exosome biogenesis and secretion is not fully understood but is a very tightly regulated process governed by multiple signaling molecules that may be unique for each type of cells. The origin of exosomes is a result of late endosome inward budding of multivesicular bodies (MVBs) that contain selective intraluminal contents. The process is mediated by three independent pathways: the endosomal sorting complex required for transport (ESCRT)-dependent pathway, the tetraspanin-dependent pathway, and the ceramide-dependent pathway [16, 30]. The MVBs can either be degraded in the lysosome or be secreted in the form of exosomes into the extracellular space. The secretion of exosomes is mainly controlled by the proteins in the exosomal membrane, including RAB GTPase proteins and RAB effector molecules [30, 35–37]. Furthermore, exosomal secretion also can be affected by other factors, such as intracellular Ca^{2+}, p53, microenvironmental pH, and expression of heparanase [38–40]. After secretion, the exosomes will migrate to the recipient cells, but this process has not yet been explained. In recent studies, uptake and internalization of exosomes by recipient cells have been revealed to depend on three dependent mechanisms: (1) surface receptor-ligand interaction, (2) internalization through direct fusion, and (3) internalization through endocytosis [35, 41–43]. Finally, the contents transferred by exosomes can influence the function of the recipient cell (Fig. 2).

3 Isolation, detection, and analysis of exosomes

Based on the intended downstream use, isolation of exosomes from conditioned cell culture medium or body fluids such as plasma can be completed by different methods (Table 2). However, there is currently limited information on optimizing efficient methods for extraction of pure and high-quality exosomes. Among these methods, the most popular are ultracentrifugation and the affinity isolation.

At present, exosomes can be detected through different methods in nearly all human body fluids, including in blood, saliva, and urine [48]. Transmission electron microscopy, Western blot, and flow cytometry are the most commonly applied methods for exosome detection (Table 3). The marker proteins for exosomal detection include membrane-associated proteins (tetraspanins, including CD9, CD63, CD81, and CD82), ESCRT-associated proteins (Tsg101 (tumor susceptibility gene) and Alix), cytoplasmic proteins (Hsp70 (heat shock protein) and Hsp90), adhesion molecules (Integrins), and membrane transport and fusion proteins (Annexins) [30, 53, 54]. The concentration of exosomes only can be indirectly and inaccurately measured by measurements of flow cytometry, resistive pulse sensing, or nanoparticle-tracking analysis. Quantitative real-time PCR, nucleic acid sequencing, Western blot, and ELISA are used for the analysis of nucleic acid and protein contents in exosomes.

4 Roles of exosomes in sarcoma

The main function of exosomes is to transport bioactive molecules, including proteins, DNA, mRNA, and non-coding RNAs from donor cell to recipient cell, leading to the exchange of genetic information and reprogramming of the recipient cells. More recently, exosomes have been identified to be highly secreted by different sarcoma cells. Increasing evidence suggests that exosomes play important roles in the tumorigenesis, growth, progression, metastasis, immunotherapy, and drug delivery of different sarcomas, including osteosarcoma, Ewing sarcoma, and fibrosarcoma (Table 4).

4.1 Tumorigenesis

Tumorigenesis is the process of transformation of normal cells into cancer cells. The process features a series of changes at the genetic, epigenetic, and cellular level that finally reprogram cells to form a malignant mass. Cancer

Table 1 Details of different extracellular vesicles

Vesicle type	Intracellular origin	Size (nm)	Electron microscopy	Marker proteins	Ref.
Exosomes	Endosomes	30–100	Cup/dish shape	✓ Membrane-associated proteins: tetraspanin (CD9, CD63, CD81, CD82) ✓ Endosomal sorting complex required for transport-associated protein: Tsg101, Alix ✓ Cytoplasmic proteins: Hsp70, Hsp90 ✓ Membrane transport and fusion proteins: Rab GTPases, Annexins	[16–21]
Ectosomes	Plasma membrane	50–200	Round shape	CR1, C1q, ARF6	[17, 22]
Microvesicles	Plasma membrane	100–1000	Irregular shape	Integrins, Selectins, CD40 ligand	[16, 18, 19]
Apoptotic bodies	Plasma membrane	1000–5000	Heterogeneous	Histones, TSP, C3b, Annexin V, Caspase 3	[19, 23, 24]
Oncosomes	Plasma membrane	1000–10,000	Round shape	ARF6, CK18, GAPDH	[18, 25, 26]
Migrasomes	Plasma membrane	500–3000	Not identified	TSPAN4	[27]
Giant vesicles	Plasma membrane	3000–42,000	Not identified	Not identified	[28]

Tsg tumor susceptibility gene, *Hsp* heat shock protein, *TSP* tetraspanin

cells actively secrete exosomes that may contribute to tumorigenesis. Current evidence suggests that exosomes participate in many key tumor-promoting processes in different cancers, including in Ewing sarcoma and rhabdomyosarcoma.

It is well known that both CD99 and EWS-FLI1 are important in the oncogenesis and cellular differentiation of Ewing sarcoma. A study on whether the tumorigenesis of Ewing sarcoma cells could be reversed back to normal differentiation showed that CD99 and EWS-FLI1 possess antagonistic functions on Ewing sarcoma cell differentiation through NF-kB signaling, which is regulated by the miR-34a-induced Notch pathway [56]. Exosomes isolated from human Ewing sarcoma cell culture medium through ultracentrifugation or synthetic polymer-based precipitation were verified by positive RAB5B, CD63, and CD81 markers through Western blot [56]. Meanwhile, CD99 was detected in exosomes from Ewing sarcoma cells [56]. Interestingly, the contents in exosomes could be controlled by their parental cells. In CD99-silenced Ewing

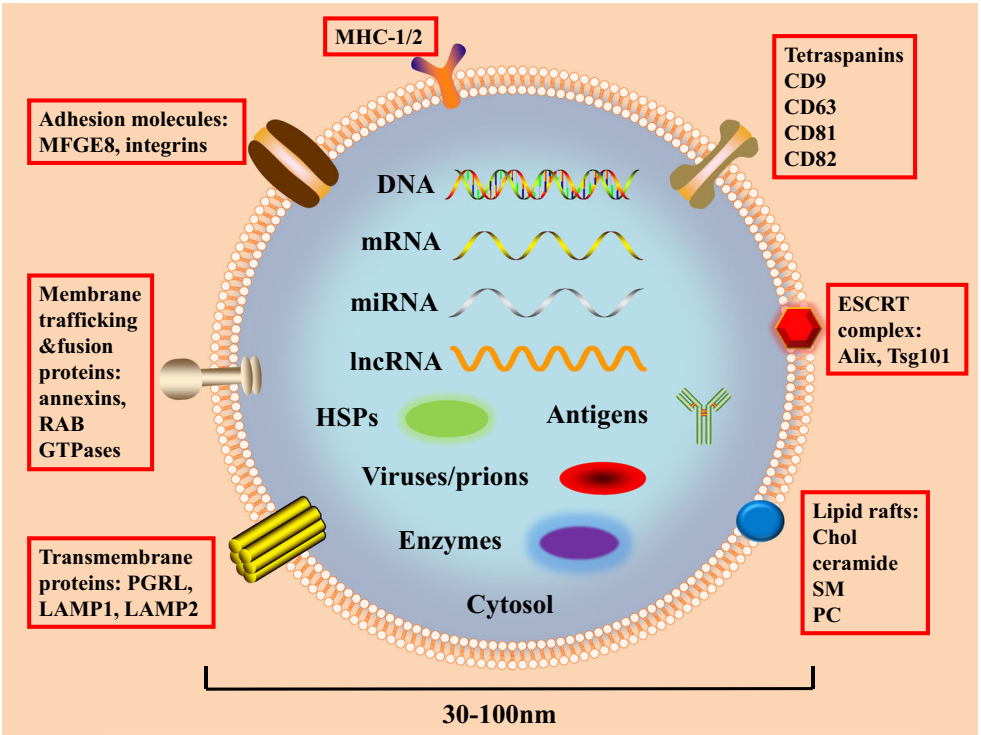

Fig. 1 Exosomes contain various molecular constituents of their parental cell, including proteins (CD9, CD63, and CD81), DNAs (single- and double-stranded), and RNAs (mRNA, miRNA, and lncRNA)

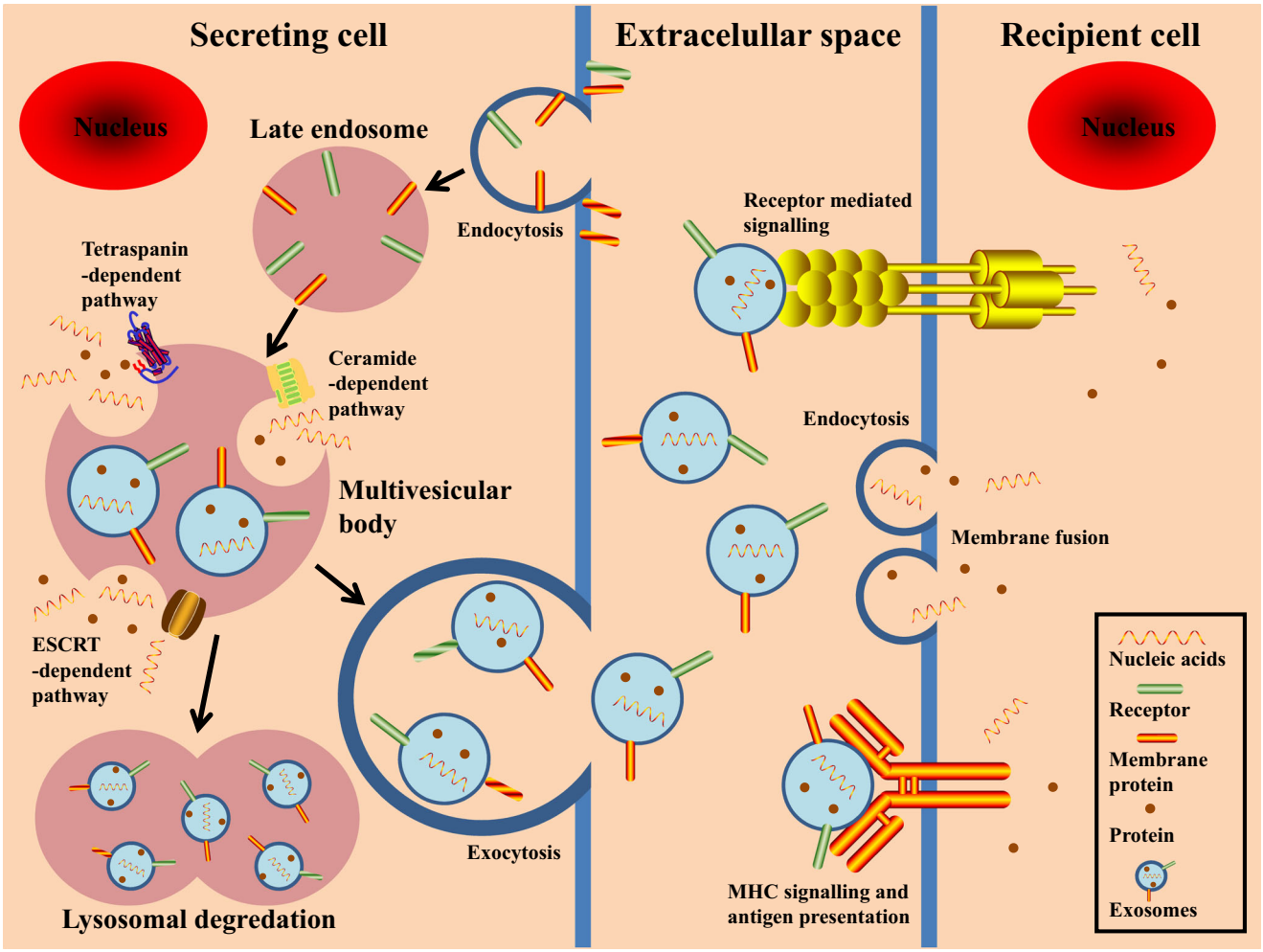

Fig. 2 The contents transferred by exosomes can influence the function of the recipient cell

sarcoma cells which was compared with normal Ewing sarcoma cells, lack of CD99, higher level of miR-34a, and lower levels of Notch 1 and Notch 3 were simultaneously detected in the secreted exosomes [56]. From another point of view, the exosomes could conversely impact their parental cells. It was demonstrated that exosomes from CD99-silenced Ewing sarcoma cells were capable of influencing the recipient Ewing sarcoma cells by mimicking CD99 silencing and inducing neural differentiation in recipient Ewing sarcoma cells [56]. The mechanism of the mimetic CD99 silencing in recipient cells was through downregulation of NF-kB signaling by increasing miR-34a after internalization of exosomes from the CD99-silenced Ewing sarcoma cells [56]. In addition, the exosome internalization by recipient cells was indirectly confirmed owing to the different CD99 expression in CD99-shRNA Ewing sarcoma cells after respective exposure to the exosomes derived from parental and CD99-silenced Ewing sarcoma cells [56]. Thus, it has been suggested that exosomes can be secreted by Ewing sarcoma cells and those important messages contained in exosomes could actively participate in the tumorigenesis of Ewing sarcoma.

4.2 Intercellular communication

Intercellular communication is significant for the vital functions of tumor cells. Traditional models of intercellular communication include transfer of secreted molecules and direct contact; however, recent studies have found that exosomes play important roles in cell-to-cell communication in different tumors. During their course, exosomes have the potential to deliver a number of bioactive molecules, surface receptors, and genetic information from donor cells to recipient cells.

From these findings, exosomes were used to study the cell-to-cell communication between human glioblastoma cells and human fibrosarcoma cells. Exosomes from human glioblastoma cells (U251-MG and U251) were shown to be internalized by human fibrosarcoma cells HT-1080, other tumor cells, and normal astrocytes through confocal microscopy analysis and

Table 2 Methods for exosome isolation

Methods	Details of procedure	Advantages	Limitations	Ref.
Ultracentrifugation/differential centrifugation/density gradient centrifugation/sucrose cushion centrifugation	1. Differential centrifugation: four different centrifugation steps (300×*g* for 10 min, 2000×*g* for 10 min, 10,000–20,000×*g* for 30 min, and 100,000–200,000×*g* for 70 min or longer) 2. Loaded onto sucrose and iodixanol density gradient/cushion 3. Centrifugation (75,000×*g* or greater, 75 min overnight) 4. Diluted with PBS 5. Centrifugation (100,000×*g*, 30 min–2.5 h)	✓ "Gold standard" for isolation of exosomes ✓ Medium yield ✓ High purity for iodixanol density gradient ✓ Low cost ✓ No chemical additives	✘ Efficacy depends on the physicochemical properties of a sample ✘ Low purity for some kinds of centrifugation	[44–46]
Affinity isolation	1. Fuse an affinity tag (membrane antibodies, biospecific peptide, or proteoglycan affinity reagents) with either magnetic or agarose beads 2. Eluted using appropriate buffers	✓ Enables examination of selected subpopulations of exosomes ✓ High purity due to specific marker ✓ Easy coupling with other methods to further research	✘ Low yield ✘ Antibody contamination ✘ Limited possibility of isolation from large volumes ✘ Elution buffers can contain incompatible components ✘ Expensive	[44–46]
Size exclusion chromatography	1. Low-speed centrifugation to remove larger objects 2. Filtration to pre-concentrate the extracellular vesicles 3. Size exclusion chromatography operated under gravity or by inexpensive pumps 4. Centrifugation (100,000×*g*, 1 h or longer) to yield exosomes	✓ High purity ✓ Short time ✓ Low cost ✓ No chemical additives	✘ Pre-concentrate filters cause deformation and rupture of extracellular vesicles ✘ Low yield ✘ Significant dilution of the final sample ✘ Low sample throughput	[44–46]
Synthetic polymer-based precipitation	1. Combination of precipitation solution consisting of PEG (polyethylene glycol) with biofluid and incubation overnight at 4 °C 2. Centrifugation at low speed	✓ High yield ✓ No special equipment required	✘ Polymer particles contamination ✘ Efficiency influenced by reagent's manufacturer	[45–47]
Membrane filtration	1. Stirred ultrafiltration cells 2. Ultrafiltration spin columns/tubes operated using low centrifugal force	✓ High purity	✘ Low yield	[45]
Microfluidic technologies	1. Immunoaffinity 2. Sieving 3. Trapping exosomes on porous structures	✓ Diagnostic purpose ✓ High efficiency ✓ High purity ✓ Minimal processing time	✘ Low yield	[47]

flow-cytometric analysis [59]. Notably, the internalization of exosomes by HT-1080 cells manifested more efficiently, and the phenomenon of saturation between donor cells and recipient cells of exosomal uptake was observed [59]. Then, in order to further explore the mechanism of exosomal uptake, scientists removed all the surface proteins from exosomes using trypsin and found that the uptake of exosomes by recipient cells was not impacted, which indicated that surface protein ligands on exosomes was not the only method of exosome internalization [59]. Considering the lipid bilayer structure of exosome and the specificity of exosomal lipid components according to different parental cells, lipid components were suspected as another means of exosome internalization. Comparing the lipid components of the extracellular vesicles derived from tumor cells and from astrocytes, the lipid components were proved significantly different [59]. The result implied that the lipids of exosomes were another possible mechanism of cell-to-cell communication. Taken together, these data demonstrate that exosomes are essential for intercellular interaction not only between tumor cells and normal cells but also between different tumors. Furthermore, apart from surface proteins, exosomes can also induce intercellular communication through the lipids on the membrane of exosomes.

4.3 Modulation of the microenvironment

Increasing evidence indicates that in addition to cancer cells themselves, dysfunction in the tumor microenvironment can be crucial for carcinogenesis. Targeting the microenvironment of tumor cells has been proposed as a novel strategy for cancer treatment. New data point out that exosomes from tumors can modulate the microenvironment not only by reprogramming surrounding normal

stromal cells but also by promoting intercellular communication within tumor cells themselves. These microenvironment modifications by tumor exosomes appear to be important for tumor growth and invasion.

As the most common tumors in gastrointestinal tract, gastrointestinal stromal tumors (GISTs) arise from interstitial cells of Cajal (ICCs) and their precursors and present KIT and PDGFRA mutations most commonly [61, 63, 64]. In one recent study, GISTs were reported to secrete exosomes to modify the microenvironment. Exosomes derived from GISTs-T1 cells were isolated through ultracentrifugation and evaluated by the size and positive exosomal markers (CD63, CD9, ALIX, TSG101, flotillin, and Annexin 1) through phase contrast electron micrography, nanoparticle-tracking analysis, and immunoblot analysis [61]. Interestingly, to examine whether more information could be derived from malignant exosomes, exosomes from GIST-T1 cells were compared with exosomes from retrovirally transformed human myometrial smooth muscle cells (ULTR). The exosomes from GIST-T1 cells were confirmed to contain more substances due to their greater density, size, and protein contents [61]. At the same time, oncologic KIT was manifested to be uniquely contained at the surface of exosomes from GISTs-T1 cells and on the membrane of GISTs-T1 cells, whereas exosomes from ULTR cells were negative for KIT [61]. This result once again verified that the KIT mutation was closely and distinctively related with GISTs. To explore the mechanism of microenvironment modification by exosomes, interactions between exosomes from GIST-T1 cells and the ULTR cells were studied. Through confocal microscopy and flow cytometry, GIST-derived exosomes were determined to be efficiently internalized by ULTR cells. Simultaneously, the adhesion ability of ULTR cells to extracellular matrix was observed to be improved after internalization of exosomes from GIST-T1 cells [61]. Furthermore, after exosomal internalization by ULTR cells, the expressions of ICC-associated transcripts and proteins in ULTR cells were induced [61]. Significantly, however, the ICC-associated transcripts and proteins were not directly transferred into ULTR cells by the exosomes, which demonstrated that exosomes could promote transformations of the surrounding normal stromal cells [61]. More impressively, the ICC-like ULTR cells transformed by the exosomes from GIST-T1 cells exhibited secretion of MMPs (metalloproteinases), including MMP1, which improved the invasion of GIST-T1 cells [61]. Hence, exosomes can be secreted by GIST cells, and GISTs are able to influence the surrounding normal cells through exosomes and accept the possible feedback from the remodeled surrounding cells. This preliminary study may be a step toward further research and understanding of the role of exosomes in the microenvironment modification of sarcoma.

4.4 Metastasis

As one of the hallmarks of malignant tumors, metastasis is a complicated process. It is essential to optimize a microenvironment suitable for tumor metastasis both locally and distantly [65]. Emerging evidence suggests that cancer-derived exosomes can selectively sort and transfer metastatic messages to a recipient cell and modify the local environment for metastasis, thus playing significant roles in tumor metastasis.

To date, only one study has described the existence and secretion of exosomes from osteosarcoma cells. Firstly, scientists distinguished a group of different osteosarcoma cell lines depending on their metastatic characteristics through *in vivo* and *in vitro* tests [66]. Among these metastatic osteosarcoma cell lines, KHOS cells were chosen as the exosomal donor cells because of their high expression of the uPA-uPAR axis (uPA and plasma membrane-associated uPAR), which was proved to be exclusively associated with the metastatic behavior of osteosarcoma independent of Ras status [66]. Exosomes from KHOS cells were isolated through ultracentrifugation and confirmed by their particle diameter range (30–90 nm) and CD63 protein-positive staining through dynamic light scattering and Western blot [66]. Meanwhile, uPA was detected both in exosomes and in the conditioned medium from tumors as the exosomal and soluble forms, respectively [66]. Although further research about the relationship of the exosomes and metastasis in osteosarcoma has not been completed, it is still reasonable to conclude that exosomes are potential to influence metastatic behavior of osteosarcoma cells locally and at distant sites (Table 5).

As an extracellular enzyme, membrane type 1 matrix metalloproteinase (MT1-MMP, MMP-14) has been considered to play important roles in cell migration, matrix remodeling, and tumor invasion. It can be transferred through endocytosis from the cell surface to late endosomes, which occurs in the biogenesis of exosomes [70, 71]. In order to explore whether endosomal MT1-MMP could be released out of cells in the form of exosomes, extracellular vesicles isolated from human fibrosarcoma cells through sucrose density gradient centrifugation were described as exosomes through immunoblotting and transmission electron microscopy analysis [67]. In fibrosarcoma cells, full-length (active) and proteolytically processed (inactive) forms of MT1-MMP were both proved to be contained in the exosomes [67]. Moreover, in fibrosarcoma cells, the exosomal MT1-MMP was determined to be functionally active due to activating pro-MMP-2 and degrading type 1 collagen and gelatin [67]. Thus, we can confirm that

Table 3 Methods for detecting exosomes

Methods	Range of analysis	Advantages	Limitations	Ref.
Nanoparticle-tracking analysis	Size distribution (50 nm–2 μm) Concentration (10^7–10^9 particles/ml)	✓ Visual confirmation ✓ Phenotype study ✓ High throughput	✗ Size >70 nm (fluorescent-nanoparticle-tracking analysis, >50 nm)	[45, 49, 50]
Dynamic light scattering	Size distribution (5 nm–1 μm)	✓ Visual confirmation ✓ High throughput	✗ Requires careful sample preparation to delete large particles ✗ Not amenable to molecular labeling	[45, 49, 50]
Flow cytometry	Size distribution (100 nm–1 μm) Concentration (10^6–10^9 particles/ml)	✓ Measures individual exosomes ✓ Measures multiple surface markers of exosomes ✓ Imaging flow cytometry allows for visual confirmation ✓ Phenotype study ✓ High throughput	✗ Size >100 nm ✗ Expensive for imaging flow cytometry	[45, 49, 50]
Electron microscope	2 nm–μm range	✓ Gold standard ✓ Visual confirmation ✓ Phenotype study	✗ Transmission electron microscope: sample fixation induces shrinkage of exosome structure and non-optical method ✗ Time consuming ✗ Expensive	[45, 49, 50]
Atomic force microscopy	0.5 nm–μm range	✓ Visual confirmation	✗ Time consuming	[45, 49, 50]
Resistive pulse sensing impedance-based FCM	Size distribution (40 nm–1 μm) Concentration (10^7–10^{10} particles/ml)	✓ High throughput	–	[45, 49, 50]
Western blot	–	✓ Depends on multiple surface markers of exosomes	✗ Indirect detection	–
Extracellular vesicle array	–	✓ No enrichment or purification before analysis ✓ Depends on a cocktail of surface markers of exosomes ✓ Phenotype study	✗ Indirect detection	[51, 52]

exosomes are one mode for MT1-MMP secretion by sarcoma cells to increase metastasis (Table 5).

In another study on rhabdomyosarcoma, data showed that lysosomal exocytosis, which is positively correlated with the release of exosomes, could be induced to transform tumor cells to an invasive and drug-resistant phenotype by downregulation of NEU1 (target of sialidase) and accumulation of LAMP1 (sialylated membrane glycoprotein) [68]. To determine the mechanism of NEU1-LAMP1 controlling lysosomal exocytosis in sarcoma, human rhabdomyosarcoma cell lines RH41 with higher-NEU1 and lower-LAMP1 expression and RH30 with lower-NEU1 and higher-LAMP1 expression were examined and compared [68]. In both RH41 and RH30 cells, exosomes were isolated through ultracentrifugation and detected by positive expression of exosomal markers flotillin-1, CD81, syndecan-1, and syntenin-1; however, more exosomes were observed to be secreted by RH30 cells [68]. In RH30 cells, more lysosomes were found tethered to or docked at the plasma membrane and showed a stronger trend for exocytosis [68]. For further confirmation, scientists knocked down and overexpressed the NEU1 or LAMP1 genes, respectively, in RH41 and RH30 cells and indicated that lysosomal exocytosis and release of exosomes were both enhanced by silencing NEU1 or upregulating LAMP1 [68]. Intriguingly, communication between exosomes and cells was observed. The invasive capacity of RH41 cells was increased by culturing in the presence of exosomes from the RH30 cells, but $RH30^{shLAMP1}$ exosomes were unable to show the same effect on RH41 cells [68]. To further explore the mechanisms of tumor progression under the conditions of NEU1 downregulation and LAMP1 upregulation in sarcoma, a gene expression array of sarcomas was performed. The results showed that MYH11 and Myosin-11 were closely correlated with NEU1 and LAMP1, respectively, and a synergistic mode of action was observed [68]. In addition, increased lysosomal exocytosis and exosomal release were together shown to strengthen the invasive capability of both human and mouse sarcoma cells and degrade the surrounding extracellular

matrix [68]. Overall, this study verifies the release of exosomes from rhabdomyosarcoma and supports a role of exosomes in influencing the metastasis of rhabdomyosarcoma (Table 5).

4.5 Immunotherapy

The immune system is very important in protecting humans from tumors. Tumor antigens can induce T cell activation, which can lead to either immune response or tolerance, but not tumor eradication. Since exosomes contain the antigens from cancer cells and may influence the immune response, these could be a possible target for immunotherapy.

Recently, in order to study and improve endogenous antitumor immune responses in sarcomas, scientists examined and compared the immunogenicity of antigens in different secretion modalities. Firstly, to generate the different tumor cell lines secreting either soluble or membrane-associated OVA (chicken egg ovalbumin), murine fibrosarcoma tumor cell lines MCA101 were transfected by different plasmids that were constructed directly by antigen OVA (MCA/sOVA cells) or by the fusion complex of the antigen OVA and the C1C2 domains of lactadherin mediating lactadherin binding to phosphatidylserine-exposing membrane vesicles (MCA/OVAC1C2 cells) [57]. Exosomes from these two transfected cell mediums were isolated through ultracentrifugation and defined by the typical cup-shaped morphology, size (60–110-nm diameter), and positive expression of tetraspanin CD9, HSP70, and ESCRT protein TSG101 through electron microscopy and Western blot [57]. Successfully, soluble OVA and exosome-bound OVA were strongly detected in the supernatant from MCA/sOVA cells and the exosomes from MCA/OVAC1C2 cells, respectively [57]. To further examine the function of the antigens in exosomes, *in vivo* testing of a mouse model was used. The antigens in the exosomes were demonstrated to elicit more efficient antitumor immune response and inhibit the growth of tumors [57]. Simultaneously, antigens in exosomes induced a greater immunogenic effect than those antigens in soluble form [57]. Moreover, the antigen in exosomes distributed by vaccination was observed to have inhibition function on the progression of tumor in mice [57]. Hence, exosomes are verified to be secreted by fibrosarcoma and could be a potential novel and more efficient method of inducing antitumor immune responses.

More recently, further research focused on whether the antitumor immune response was dependent on the localization of the antigens. The same methods were applied to artificially generate the different tumor cell lines secreting either soluble or membrane-associated OVA and detect exosomes in the according conditioned medium [57, 58]. Compared with soluble antigens, the antigens derived from exosomes were shown to induce a stronger CD8+ T cell response, more antigen-specific antibodies, help from CD4+ T cell, and a reduction in the percentage of the CD4+ regulatory T cells, which can negatively influence the immune response [58]. Moreover, after therapeutic interference with cryoablation in tumors, only antigens from exosomes could boost CD8+ T cell immune response [58]. Thus, the location of antigens was determined to be very important to induce an immune response, and this greater immune response induced by exosomal antigens has been confirmed.

In addition, one study aimed to explore the protein profiles of the exosomes and their alterations under drug effects. The exosomes from human synovial sarcoma cell line SW982 cells were isolated through synthetic polymer-based precipitation and authenticated by their size (30–100 nm) and positive exosome-associated markers (ALIX and HSP70) through transmission electron microscopic analysis and Western blot [62]. The protein profiles of the exosomes from SW982 cells could be altered under the different influences from salazosulfapyridine (SASP) and methotrexate (MTX) respectively and synergistically [62]. Meanwhile, IL-1β was shown to not only influence the protein profiles of exosomes from SW982 cells but also be inhibited by SASP and MTX [62]. Intriguingly, the protein content in exosomes was approximately one third of that in SW982 cells, and the changes of protein profiles between the exosomes and SW982 cells were observed to be distinctly different after the SASP+MTX treatment, which implicated that the contents of exosomes are not a simple reproduction of the parental cells [62]. More importantly, two functional groups of proteins were observed in exosomes named the immune system-related proteins, which include immune suppressor protein Rab-7b, protein Ran and SH2 domain containing protein 1B, and the oxidative stress-related proteins [62]. These two groups of proteins in exosomes were verified probably as the targets of SASP and/or MTX under the conditions with/without IL-1β. Taken together, the study indicated that exosomes can be secreted by synovial sarcoma cells, the proteins in the exosomes are significant for subsequent functions, and the immune system-related proteins can be regarded as possible immunotherapy targets in the future.

4.6 Sarcoma biomarkers

The proteins and nucleic acids in exosomes have been regarded as ideal diagnostic biomarkers and prognostic indicators because of their wide distribution and critical roles in nearly all activities of tumors. The amount of exosome secretion has been proven to be positively correlated with the malignancy grade of some tumors [72].

In a preclinical study investigating suitable candidates as diagnostic biomarkers for Ewing sarcoma, exosomes were first observed to be secreted by Ewing sarcoma cells (Table 5). Exosomes from Ewing sarcoma cells were isolated through ultracentrifugation and verified based on their 30–

Table 4 Details of some roles of exosomes in different sarcomas

Tumors	Exosome origin	Exosome marker	Exosome content	Exosome targets	Exosome functions	Ref.
OS	Human bone marrow-derived MSCs	CD81, Flotillin-1, Alix	miR143	Human osteosarcoma cell line 143B	✓ Delivery system ✓ Tumor proliferation ✓ Tumor migration	[55]
ES	Human ES cells	RAB5B, CD63, CD81	CD99, miR-34a	miR-34a/Notch/NF-kB pathway	Tumorigenesis	[56]
FS	Murine FS cells	CD9, Hsp 70, Tsg 101	OVA fusion with C1C2 domain of MFG-E8/ lactadherin	✓ CD8+ T cell response	Tumor-related immune response	[57]
FS	Murine FS cells	CD9, Hsp 70, Tsg 101	OVA fusion with C1C2 domain of MFG-E8/ lactadherin	✓ Activation of CD8+ T cells ✓ Proliferation of CD4+ cells IgG1 antibody response	Tumor-related immune response	[58]
FS	Human glioblastoma cells	CD63, Tsg 101	Lipid compositions: PE, SM, PI, PS, PC, Chol	–	Cell interactions	[59]
RS	Human RS cells	CD63	EV71 RNA, capsid protein VP1	–	✓ Delivery system ✓ Virus infection	[60]
GISTs	Human GIST-T1 cells	CD63, CD9, Alix, Tsg 101, Flotillin, Annexin 1	KIT	✓ AKT ERK1/2	Microenvironment modification	[61]
SS	Human SS cells	Alix, Hsp 70	Immune- and oxidative stress-related proteins	–	Tumor-related immune response	[62]

Abbreviations: *OS* osteosarcoma, *MSCs* mesenchymal stem cells, *miR* microRNA, *ES* Ewing sarcoma, *NF-kB* nuclear factor-kB, *FS* fibrosarcoma, *Tsg* tumor susceptibility gene, *MT1-MMP* membrane type 1 matrix metalloproteinase, *OVA* chicken egg ovalbumin, *Hsp* heat shock protein, *PE* phosphatidylethanolamine, *SM* sphingomyelin, *PI* phosphatidylinositol, *PS* phosphatidylserine, *PC* phosphatidylcholine, *Chol* cholesterol, *RS* rhabdomyosarcoma, *EV* enterovirus, *GISTs* gastrointestinal stromal tumors, *KIT* protein tyrosine kinase, *SS* synovial sarcoma

100-nm size and positive CD63 and CD81 staining by electron microscopy and flow-cytometric assessment, respectively [69]. To prove that intact mRNA of Ewing sarcoma cells was contained in exosomes, 12 selected genes significantly overexpressed and specific for Ewing sarcoma were all detected in the exosomes derived from Ewing sarcoma cells [69]. Moreover, it was shown that exosomes could protect the RNA contents against the RNase in human plasma; therefore, the circulatory stability of functional mRNA in the exosomes promote the possibility of tumor biomarkers in circulation [69]. To further determine the potentially sensitive biomarkers for exosomal detection of Ewing sarcoma, five genes of the 12 potential biomarkers transcripts in exosomes were selected because of their higher specificity [69]. Finally, to explore the function of exosomes derived from Ewing sarcoma cells, RNA in exosomes and their parental cells were isolated for microarray analysis. The results showed that over 1200 transcripts were highly altered in exosomes derived from Ewing sarcoma cell lines, and the highly enriched transcripts in exosomes had the potential to become the means for

Table 5 Summary of the roles of exosomes in metastasis and in diagnostic biomarker of sarcomas

Tumors	Exosome origin	Exosome marker	Exosome content	Exosome targets	Exosome functions	Ref.
OS	Human metastatic OS cells	CD63	uPA	uPAR	Tumor metastasis	[66]
FS	Human FS cells	CD9, Tsg 101, β-1 integrin	MT1-MMP (MMP-14), β-1 integrin	✓ Pro-MMP-2 ✓ Type 1 collagen ✓ Gelatin	Tumor metastasis	[67]
RS	Human RS cells	CD81, Flotillin-1, Syndecan-1, Syntenin-1	LAMP1, NEU1	Myosin-11	Tumor metastasis	[68]
ES	Human ES cells	CD63, CD81	mRNAs	–	✓ Diagnostic biomarker ✓ Cell interaction	[69]

Abbreviations: *OS* osteosarcoma, *uPA* urokinase plasminogen activator, *uPAR* urokinase plasminogen activator receptor, *FS* fibrosarcoma, *Tsg* tumor susceptibility gene, *MT1-MMP* membrane type 1 matrix metalloproteinase, *RS* rhabdomyosarcoma, *ES* Ewing sarcoma, *mRNA* messenger RNA

intercellular and cell-microenvironment communication [69]. Therefore, the specific genes in exosomes from Ewing sarcoma should be further expanded to develop our understanding of the biomarkers for malignancy diagnosis and follow-up.

4.7 The potential role of exosomes in sarcoma prognosis

Exosome contents may include tumor-associated proteins, enzymes, growth factors, bioactive lipids, miRNAs, and DNA sequences. Therefore, exosomes present in blood or other biofluids offer unprecedented, distant, and non-invasive access to critical molecular information about the status of tumor cells. In addition, exosomes may provide a non-invasive means to assess cancer pathogenesis, progression, and treatment outcomes. Recently, the exosome-shuttling miRNA [73–75], lncRNA [76], and proteins [77] have been demonstrated to be closely related to the prognosis of a variety of tumors, including esophageal cancer, ovarian cancer, lung cancer, bladder cancer, and breast cancer [73–78]. These investigations demonstrate the applicability of exosomes as biomarkers of cancer prognosis to sarcoma patients. However, the potential role of exosomes in the prognosis in different sarcomas is currently unknown. It is hoped that in the future, analysis of tumor-specific biomarkers within sarcoma-derived exosomes may not only offer the diagnosis but may also give a convenient means of monitoring the outcome of treatment in sarcoma patients.

4.8 Targeted delivery system

Recently, pharmaceutical vehicle delivery systems have been proposed for tumor therapy. Although the encapsulation of doxorubicin in liposomes (Doxil®) and paclitaxel in protein-based nanoparticles (Abraxane®) have been applied successfully, exosomes still exhibit some advantages, including the following: (1) exosomes have a wide distribution and biocompatibility in the body [79], (2) exosomes can sort biogenic and synthetic substances, (3) donor cell of exosomes can determine the preferences of homing targets [80], and (4) membrane modifications of exosomes can determine the target cells [81]. The methods of using exosome as targeted delivery can be completed either by loading during biogenesis and secretion of exosomes, including loading parental cells with a drug or transfecting parental cells with therapeutically DNAs, or by directly loading drug or therapeutically DNAs into purified and naive exosomes (Fig. 3). Both lipophilic and hydrophilic substances can be loaded into exosomes through different methods [82–84]. Additionally, the donor cells of exosomes can partially influence the delivery system. The ideal donor cells can secret a large amount of exosomes, and the exosomes from ideal donor cells can not only induce non-immunogenic response but also localize to the specific recipient cells. At present, the human 12embryonic stem cell-

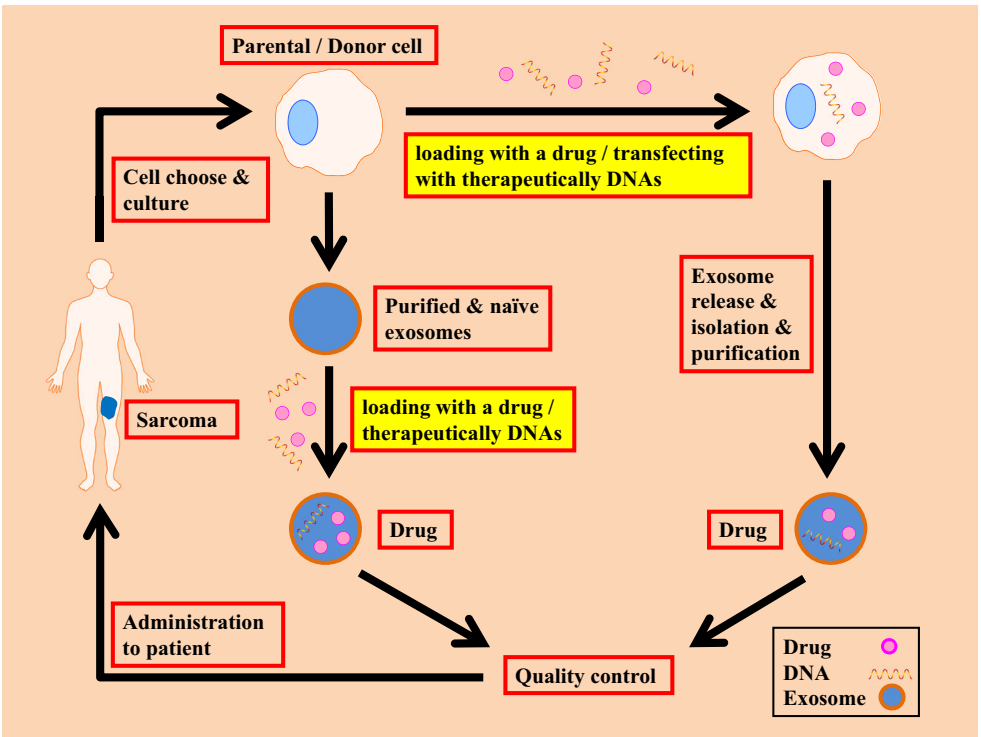

Fig. 3 The methods of using exosome as targeted delivery can be completed either by loading during biogenesis and secretion of exosomes, including loading parental cells with a drug or transfecting parental cells with therapeutically DNAs or by directly loading drug or therapeutically DNAs into purified and naive exosomes

derived mesenchymal stem cells (hESC-MSCs) have demonstrated the most benefit [85].

In one study on osteosarcoma, exosomes were applied as an effective delivery system for synthetic miR143, which is inversely related to tumorigenicity, metastasis, and chemoresistance in osteosarcoma cell lines and primary osteosarcoma tumor samples [86, 87]. MSCs were chosen as donor cells of exosomes and transfected with synthetic miR-143 through lipofection [55]. Successful transfection was confirmed based on the detection of miR-143 not only in the MSCs but also in the conditioned medium derived from MSCs. Then to prove that extracellular miR-143 could be transferred into the human osteosarcoma cell line 143B, 143B cells were cultured in the conditioned medium derived from transfected MSCs [55]. The result showed significantly higher expression levels of miR-143 in the 143B cells [55]. Meanwhile, the migration capability of 143B cells was significantly improved by extracellular miR-143 in the conditioned medium and demonstrated extracellular miR-143 dose dependence [55]. For further investigation, the mechanism of transferring extracellular miR-143 into 143B cells was explored. Exosomes from the conditioned medium of transfected and non-transfected MSCs were isolated through ultracentrifugation and verified by their size (mostly 60–75 nm) and positive expression of exosome-associated marker (CD81, flotillin-1, and ALIX) [55]. The extracellular miR-143 level was observed to be higher in exosomes than in the conditioned medium without exosomes [55]. Simultaneously, compared with non-transfected MSCs, the secretion of extracellular vesicles from transfected MSCs was higher [55]. Moreover, the migration capability of 143B cells could be improved by the conditioned medium containing exosomes, but not in the conditioned medium without exosomes [55]. These results implicated that most of the extracellular miR-143 was contained in exosomes. Intriguingly, to determine the transfer efficiency of the exosome delivery system, scientists compared the transfer efficiency of miR-143 to 143B cells between the exosome delivery system and the lipofection method. The exosome delivery system showed less toxicity and the same inhibitory effect on tumor migration but significantly lower transfer efficiency. One possible reason was that the low transfer efficiency of exosomes may be enough for the subsequent function, while the other possible reason could be that the miR-143 in exosomes might be incorporated into other complexes [55, 88]. Therefore, although exosomes were demonstrated to have the potential to be an efficient and functional delivery system, further research on the mechanism of exosomal delivery is still necessary.

In addition, exosomes can also be used as a delivery system for viral infection. In one study on the pathogenesis of hand, foot, and mouth disease (HFMD), scientists tried to explore the roles of exosomes containing enterovirus 71, which is a major pathogen of HFMD. Human rhabdomyosarcoma cells were chosen as exosomal donor cells [60]. Exosomes from the conditioned medium of enterovirus 71-infected or non-infected human rhabdomyosarcoma cells were isolated through ultracentrifugation and verified by the typical size and the positive expression of exosome-associated marker CD63 through electron microscopy and Western blot [60]. Meanwhile, the viral components, including enterovirus 71 RNA and protein VP1, were confirmed to be contained in the exosomes secreted from enterovirus 71-infected rhabdomyosarcoma cells [60]. In order to further explore the role of exosomes in transmitting viral infection, human neuroblastoma cells were transfected by exosomes from EV71-infected rhabdomyosarcoma cells and free enterovirus 71. Compared with transfection by free enterovirus 71, the viral component transmission into human neuroblastoma cells through exosomes demonstrated greater efficiency [60]. Moreover, it was demonstrated that the virus transmission mediated by exosomes could be partly protected against antibody neutralization [60]. These data once more indicate that exosomes may be a safe and effective delivery system.

5 Conclusion and future perspectives

The current rapid expansion of research on exosomes evidently shows that exosomes have many implications in cancers. This review brings together information on the roles of exosomes in sarcoma, including tumorigenesis, intercellular communication, immune response, targeted delivery system, diagnostic biomarker, and therapeutic target. Although the exact mechanisms of exosomes in sarcoma are still unclear, these studies highlight the increasing interest and development of knowledge of exosomes in sarcoma.

In order to explore of the roles of exosomes in the pathogenesis of sarcomas and open a new era for sarcoma diagnosis and therapy, future studies on exosomes may focus on the following aspects: (1) intensive understanding of biology, structure, contents, and biogenesis of exosomes; (2) establishing the functional study conditions of exosomes, which can reflect true pathophysiological conditions; (3) seeking new methods to obtain and purify a large enough number of exosomes; (4) understanding and optimizing the target delivery mechanism of exosomes; (5) finding more convenient and effective means to detect and analyze exosomes; and (6) exploiting valid ways to load exosomes with other molecules, including antitumor drugs. Using exosomes as a sarcoma biomarker and therapeutic target is already being explored, and additional anti-sarcoma applications of exosomes will be promising.

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