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# The potential of exosomes as theragnostics in various clinical situations

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## 1 Exosomes as biomarkers of diseases and therapeutic or vaccine candidates for infections

Exosomes are small intracellular membrane-based vesicles that are naturally released by eukaryotic cells into the circulation. These so-called extracellular vesicles (EV) have important functions in cell-to-cell communication, and bodily fluids display different proteins and other cellular contents such as mRNA and microRNA in healthy subjects and patients with various diseases, which can be measured as potential diagnostic markers (see [1–3]). Tumor-derived exosomes are abundant in miRNAs that may serve as tumor markers (see [4–6]). For example, the RNA contents in serum EVs of patients with glioblastoma multiforme markedly differed from those in healthy subjects (see [7, 8]), which showed its diagnostic potential as a biomarker.

The levels of EGFRvIII mRNA are increased in circulating exosomes from patients with glioblastoma multiforme, and thus it can be used as a diagnostic biomarker for this disease (see [9]). This method may serve the role of a “liquid-biopsy,” thus avoiding the need to remove tissue samples from the brain for detection of EGFRvIII protein. EGFR localized to exosome membranes also was detected as a possible diagnostic biomarker for lung cancer (see [10]). Proteoglycan glypican-1 (GP1)-positive exosomes have been detected in the serum of patients with pancreatic cancer with absolute specificity and sensitivity, distinguishing them from healthy subjects and patients with benign pancreatic disease (see [11]). Levels of GP1-positive exosomes correlated with tumor effect and survival of pre- and post-surgical patients, suggesting a valuable prognostic biomarker for pancreatic cancer. In mice, GP1-positive exosomes were reliable for detecting pancreatic intraperitoneal lesions despite negative

magnetic resonance imaging findings. Plasma exosomes derived from prostate acinar cells were investigated as new biomarkers for diagnosis of prostate cancer (see [12]). Proteomics profiling of exosomes showed potential biomarkers of the disease (see [13]). Patients who experienced recurrence of hepatocellular carcinoma after liver transplantation showed increased levels of a miRNA biomarker in serum exosomes (see [14]).

Exosomes have also been evaluated as biomarkers for various non-cancer diseases of multiple organs including the central nervous system (CNS) (see [15]), liver (see [16]), kidney (see [17]), lung (see [18]), and arteries (see [19]). In the CNS, tau-mediated neuropathology is the result of extracellular accumulation of abnormally processed tau protein (see [20]). In the M1C cell neuroblastoma tauopathy model, tau protein appeared to be spread via exosomes, and AT270 phosphorylated-tau was increased in the cerebrospinal fluid (CSF), a biomarker for early stages of Alzheimer's disease (AD). The elevated levels of AT270-phosphorylated tau in the CSF seen in mild to moderate cases of sporadic AD resulted from selective abundance of phosphorylated tau in the exosome portion of the CSF relative to total CSF tau. The increase in CSF phosphorylated tau level with attack of AD was associated with an increase in the exosome-associated portion in the CSF. In another study, levels of autolysosomal proteins (cathepsin D, lysosomal-associated membrane protein 1, and ubiquitinated proteins) in neuron-derived serum exosomes distinguished patients with pre-clinical AD from matched controls and patients with frontotemporal dementia (see [21]). Altered microRNA profiles in CSF/blood exosomes associated with neurodegenerative disorders are possible new biomarkers in the early diagnosis of AD and PD (see [22]), and the fact that exosomes can deliver siRNA offers a therapeutic potential in AD (see [23, 24]). Proteomics profiling of serum exosomes identified proteins that were abundant in patients with PD compared with healthy subjects (see [25]).

With regard to lung disease, exosomes isolated from bronchoalveolar lavage fluids of patients with asthma compared with healthy subjects showed different miRNA profiles (see [18]). Exosomes are released from the key cells implicated in asthma such as mast cells, eosinophils, dendritic cells (DCs), T-cells, and bronchial epithelial cells. These in turn can provoke the activation or repression of other asthma-related cells and stimulate allergic responses (see [26, 27]). The DC-derived exosomes (Dex, dexosome) have costimulatory molecules on their surfaces that can stimulate allergen-specific Th2 cells (see [28, 29]). The eosinophil-derived exosomes also have important roles in the modulation of asthma, and their numbers are increased in asthmatic patients (see [30, 31]). The exosomal miRNA content in patients with severe asthma was significantly different compared with healthy subjects (see [32]). The dysregulated miRNAs were associated with pathways related to airway integrity as well as being correlated with certain clinical features such as eosinophil count or FEV1 (see [33]). In a different study, the exosomal miRNA profile in patients with severe asthma was related to the TGF- $\beta$  and ErbB signaling pathway and focal adhesion (see [34]).

Exosomes may also serve as vaccines for allergic diseases (see [35]). Exosomes isolated from the bronchoalveolar lavage fluid of mice after respiratory exposure to the olive pollen allergen induced tolerance and protection against allergic sensitization in mice (see [31]).

Increased miR-192 levels in serum exosomes predicted the development of heart failure after acute myocardial infarction (see [36]). Finally, urinary exosomes have been used as starting material for diagnostic biomarkers for renal, urogenital, and systemic diseases (see [17, 37]).

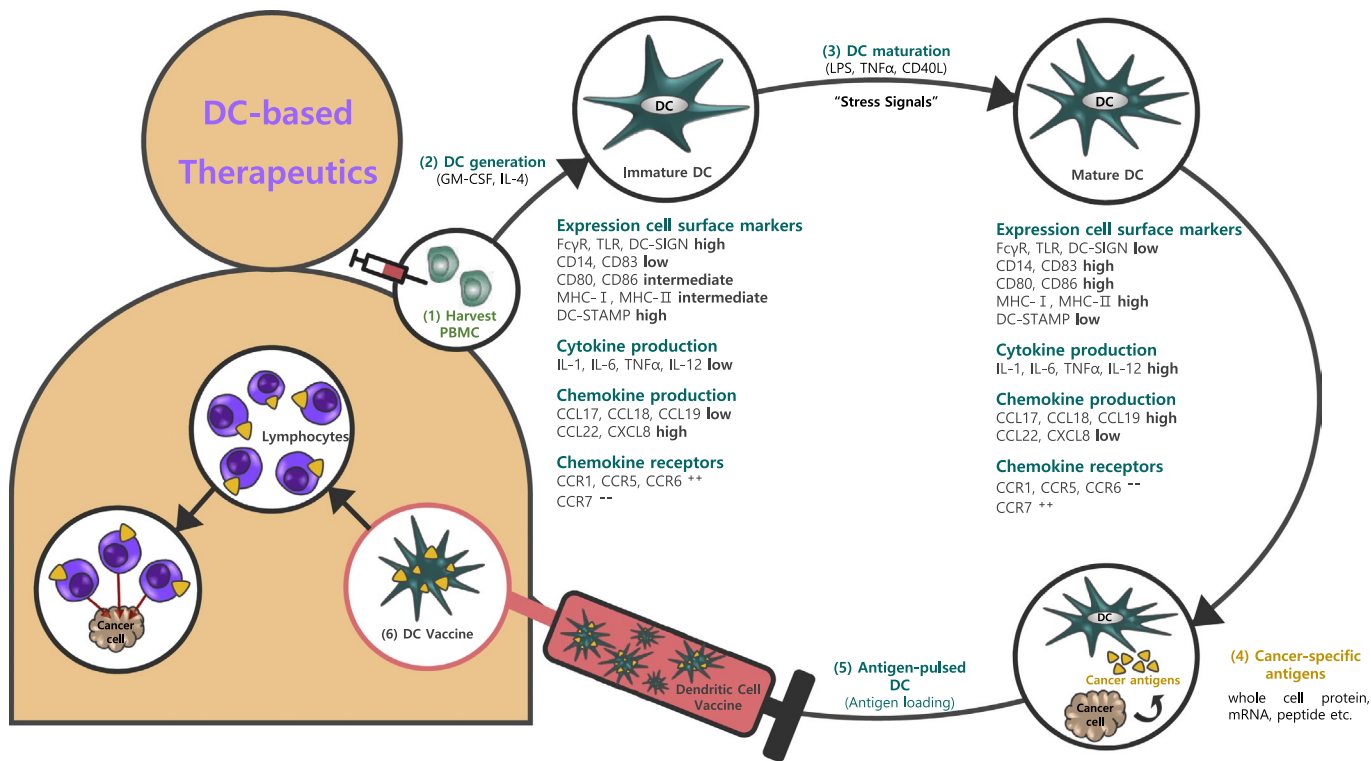
Despite the number of studies showing the relevance of EV biomarkers with diverse diseases, the results of individual studies have shown inconsistent trends. Methodological differences in EV purification may explain this contradiction (see [38]). For a given application, it is mandatory to inspect the method in terms of its sensitivity and specificity including quality control measures under well-defined settings.

Exosomes are preferential candidates for use in vaccines for infectious diseases such as toxoplasmosis, diphtheria, tuberculosis, and atypical severe acute respiratory syndrome (SARS). It has been reported that immunization using DCs with *Toxoplasma gondii* antigens (T-Ag) in healthy mice induced protection against a virulent strain of *T. gondii* after oral application, but it was difficult to obtain a sufficient amount of DCs pertinent to vaccination (see [39–41]). Murine bone marrow-derived DCs pulsed in vitro with intact diphtheria toxin (DT)-released exosomes after injection into mice showed induction of IgG2b and IgG2a responses specific for DT (see [42]). Infection with *Mycobacterium tuberculosis* excites macrophages to stimulate the release of exosomes, and it should be noted that exosomes containing *M. tuberculosis* peptide-MHC-II complexes can induce antimicrobial T-cell responses (see [43, 44]). Exosomes as vaccination materials have also been studied in SARS-related coronavirus (CoV), an infection that causes a fatal atypical pulmonary disease. Kuate et al. [35] found that exosomes with the SARS-CoV spike S protein produced neutralizing antibody titers, which was further reinforced by priming with the SARS-S exosome vaccine and then boosting with the presently applied adenoviral vector vaccine (see [35]).

Stimulating a potent and general cytotoxic T lymphocyte (CTL) immune reaction has therapeutic potential for various diseases, including viral infections. For example, inducing anti-Ebola virus (EboV)-specific CTL immunity could have benefits in both therapeutic and preventive settings (see [45]). In fact, stimulation of virus-specific CTLs has been recognized in survivors of acute EboV infections (see [46]), and virus-specific CTL immunity plays a crucial role in protection in several nonhuman primates, including macaques (see [47]). Furthermore, transfusion of CD8<sup>+</sup> T lymphocytes from mice infected with mouse-adapted EboV to naïve recipient mice defended them against EboV infection (see [48]). Consistently, a powerful CTL-related immunity response could also have pertinent therapeutic effects with influenza viruses A (Flu) (see [49]) and hepatitis C (HCV) virus infections (see [50]). Anticoli et al. [45] suggested an exosome-based vaccine platform to design exosomes in vivo with

the E7 protein of human papilloma virus (HPV). This method involves intramuscular injection of a DNA vector encoding HPV-E7 fused at the C-terminus of an exosome-anchoring Nef mutant protein (Nef<sup>mut</sup>). Human immunodeficiency virus type-1 (HIV-1) Nef<sup>mut</sup> is a 27-kDa protein (see [51]) connecting with raft microdomains at cellular membranes (see [52]). Nef<sup>mut</sup> lacks several anti-cellular effects generally caused by wild-type Nef, including CD4 down-regulation, increase of HIV-1 infectivity, PAK-2 stimulation, and MHC Class I down-regulation, and is found in exosomes at very high levels (see [53, 54]). In this alignment, the ≈11-kDa E7 protein produced both potent and effective antigen-specific CTL immunity. To establish the general application of this technology, immunogenicity studies were performed with an array of viral products of various origins and sizes including EboV, West Nile Virus NS3 and HCV NS3. All antigens were stable upon fusion with Nef<sup>mut</sup>, and were transferred into exosomes at levels compared to Nef<sup>mut</sup>. When injected into mice, DNA vectors expressing the various fusion products produced a clearly detectable antigen-specific CD8+ T cell response with sufficient cytotoxicity to kill peptide-loaded and/or antigen-expressing syngeneic cells (see [45]).

DCs are the most competent cells at presenting antigens, and are the only antigen-presenting cell able to stimulate naïve T cells, creating the adaptive immune reaction (see [55]). Indeed, we can define cancer immunosurveillance as a stage of stepwise results leading to the effective killing of cancer cells by T cells: specifically, DC capturing and processing of tumor neoantigens is the first phase, a process that depends on molecular signals such as pro-inflammatory cytokines, co-stimulatory ligands, dying tumor cells-derived molecules, and gut microbiome products (see [56]). Accordingly, potent DC-based cancer vaccinations have been researched for some time; some positive results using these technologies have emerged, such as Sipuleucel-T immunotherapy for castration-resistant prostate cancer (see [57]). However, the diverse application of DC-based cancer vaccines shows some main limitations (see [58, 59]). Fig. 1 describes DC-based immunotherapeutic strategies. Dexosome (Dex)-based cancer vaccines have recently emerged as an alternative that may overcome some of these obstacles. First, the Dex molecular component is simple to analyze, thus enabling the rigid definition of validation parameters (see [60]). Second, Dex components are more plentiful in peptide-MHC class II complexes, allowing for higher yields (see [58, 60]). Third, Dex compared with DC can tolerate longer-term frozen storage, for up to 6 months (see [58]). In addition to these merits, the immunosuppressive tumor microenvironment often inhibits antigen presentation and T cell stimulation by DCs, but this should not affect Dex (see [61, 62]). Finally, Dex are not associated with most of the risks related to the administration of viable cells, such as generation of immune dysfunction or microvascular occlusions (see [63]). Tumor peptide-pulsed Dex produced in vivo CTL priming, tumor growth repression, and tumor remission. Indeed, single intradermal injections stimulated significant tumor growth repression after a week, and 40–60% of the animals were tumor-free after 60 days (see [62]). Furthermore,



**FIG. 1** DCs-based immunotherapeutic strategies: (1) to harvest peripheral blood mononuclear cells, (2) to generate immature DCs with cytokine stimulation, (3) to mature DCs by sensing the presence of a potential pathogen (“stress signal”) via detection of PAMPs (exogenous signal) or infection-induced alteration in self-markers (endogenous signal), (4) cancer cells brought into contact with dendritic cells are consumed, thereby imprinting the dendritic cells with the cancer marker (5) to transfer activated antigen-presenting DCs back to the patients, (6) to stimulate robust anti-tumor immune effector cells such as T cells and NK cells.

these cell-free immunotherapeutic vaccines were more potent than directly administered viable DC vaccines, with which only 20% of the mice were tumor-free after 60 days. These differences may illustrate the exosomes' resistance to the immunomodulatory effects of the tumor microenvironment, which can block the ability of DCs to present antigens (see [62]). In the past decade, several successful clinical trials were performed assessing the feasibility, safety and efficacy of Dex-based cancer vaccines in patients with non-small cell lung cancer (NSCLC) (see [64]) and metastatic melanoma (see [65]), and in general the results were promising. In both trials, the patients received four doses of vaccine that consisted of autologous Dex loaded with several different MHC class II peptides. Vaccine production was shown to be practical, and the therapy was well tolerated with only minor grade 1–2 adverse events (see [64, 65]). A more recent phase II clinical trial evaluated the use of IFN- $\gamma$ -Dex, Dex derived from IFN- $\gamma$ -stimulated mature DC, as maintenance immunotherapy after the use of first-line chemotherapy in patients with advanced NSCLC (see [66]). This study showed the feasibility of production and safety of IFN- $\gamma$ -Dex, with only one of 26 patients developing a grade 3 hepatotoxicity. This trial did not show any objective tumor response among clinical outcomes, according to the Response Evaluation Criteria in Solid Tumors. However, it did show that the patients with the longest progression-free survival had a notable improvement in NK cell function after Dex treatment, showing that Dex can stimulate the NK cell arm of antitumor immunity in patients with advanced NSCLC (see [66]).

Tumor-derived exosomes also function as an antigen delivery system, capable of blocking tumor development in a CD4+ and CD8+ T cell-dependent pattern (see [67]). Because of this, cell-free vaccines based on the use of tumor-derived exosomes are another possibility for clinical application. However, the isolation of tumor-derived exosomes is inconvenient and has a low preparation efficiency, with a low yield from *in vitro* culture of the patients' tumor cells (see [68]). However, malignant effusions from patients with melanoma are rich in exosomes, which can transmit tumor antigens to DCs, which in turn stimulate tumor-specific CTL capable of an effective *in vitro* antitumor response (see [69]). A phase I clinical trial examined the effects of exosomes harvested from the ascites of patients with advanced CRC as immunotherapy, and showed that a combination of tumor exosomes with GM-CSF permitted a more effective induction of systemic anti-tumor immunity and CTL responses compared with tumor exosomes alone. The patients treated with only tumor exosomes showed no therapeutic response, while one patient with stable disease and one patient with a minor clinical response were observed in the group treated with ascites-derived exosomes combined with GM-CSF (see [68]). Despite the attempts engaged thus far, Dex-based immunotherapy as a novel cancer control remains a highly encouraging possibility. Dex are proficient mediators of immune responses and the technical simplicity of managing their immunostimulatory characteristics (via the donor DC) along with their advantages over whole cell-based applications, confirms their therapeutic promise (see [60]).



Of particular interest to this section, exosomes can serve as both promoters of tumor growth and invasion by establishment of an immunosuppressive microenvironment and as agents for cancer immunosurveillance by inducing antigen presentation and stimulating destruction of tumor cells by CD4+ (see [70]) and CD8+ (see [69]) T cells and by components of the innate immune system, such as NK cells (see [71]) (Fig. 1).

## 2 Extracellular vesicles (EVs) as a drug delivery system

The most important characteristic for a successful nanocarrier is satisfactory *in vivo* behavior. The development of EVs as delivery systems requires comprehension of their *in vivo* kinetics after administration. However, understanding of extracellular behavior, pathways of cell uptake, and subcellular paths of EVs remains obscure. EVs released by various types of cells can be found and are relatively stable in the blood circulation and biological fluids. This suggests that EVs are more slowly cleared and remain longer than synthetic nanocarriers in biological systems. However, different *in vivo* pharmacokinetic studies have shown that when EVs are injected into the circulation, they are rapidly cleared. EVs derived from B16 melanoma cells and splenocytes underwent rapid clearance and showed a very short half-life, approximately 2 min, after intravenous administration in mice (see [72, 73]). Increased levels of fetuin-A in urinary exosomes correlated with acute urinary injury (see [74]).

Exosomes and microvesicles participate in a large variety of body processes. They are carriers of concentrated genetic and proteomic information, and thus are believed to play important roles in cell-to-cell communication. Secreted vesicles can carry their messages in different ways. Firstly, they may stimulate recipient target cells via ligands expressed on their surface. For example, it has been verified that antigen-presenting exosomes derived from DC induce the T cell-mediated immune response *in vivo* (see [75]). In addition, ligand-receptor signaling via exosomes can also play a role in other regulatory processes, such as angiogenesis (see [76]), hemostasis (see [77]), cancer progression (see [78]), and metastasis (see [79]). Secondly, secreted EVs may transfer surface receptors from one cell to another by fusion with the plasma membrane of target cells (see [80, 81]). With this mechanism, HIV increase susceptibility to infection by transferring CD4 receptors from infected cells to intact cells (see [82]). EVs appear to have multiple obvious advantages, such as high delivery capacity, innate targeting properties, and low immunogenic potentiality, which position them as efficient biological delivery systems for therapeutics ranging from small molecules to macromolecular nucleic acids and proteins (see [83]) (Table 1).

Despite the development of various methodologies for EV-based delivery, a major hurdle is the lack of standardized, efficient, and reasonable approaches for isolation of EVs. Isolation methods need to be validated when considering the reproducibility, yield, purity, and functional properties of EVs for its general application. Also, there is no validated standard procedure for storage time,



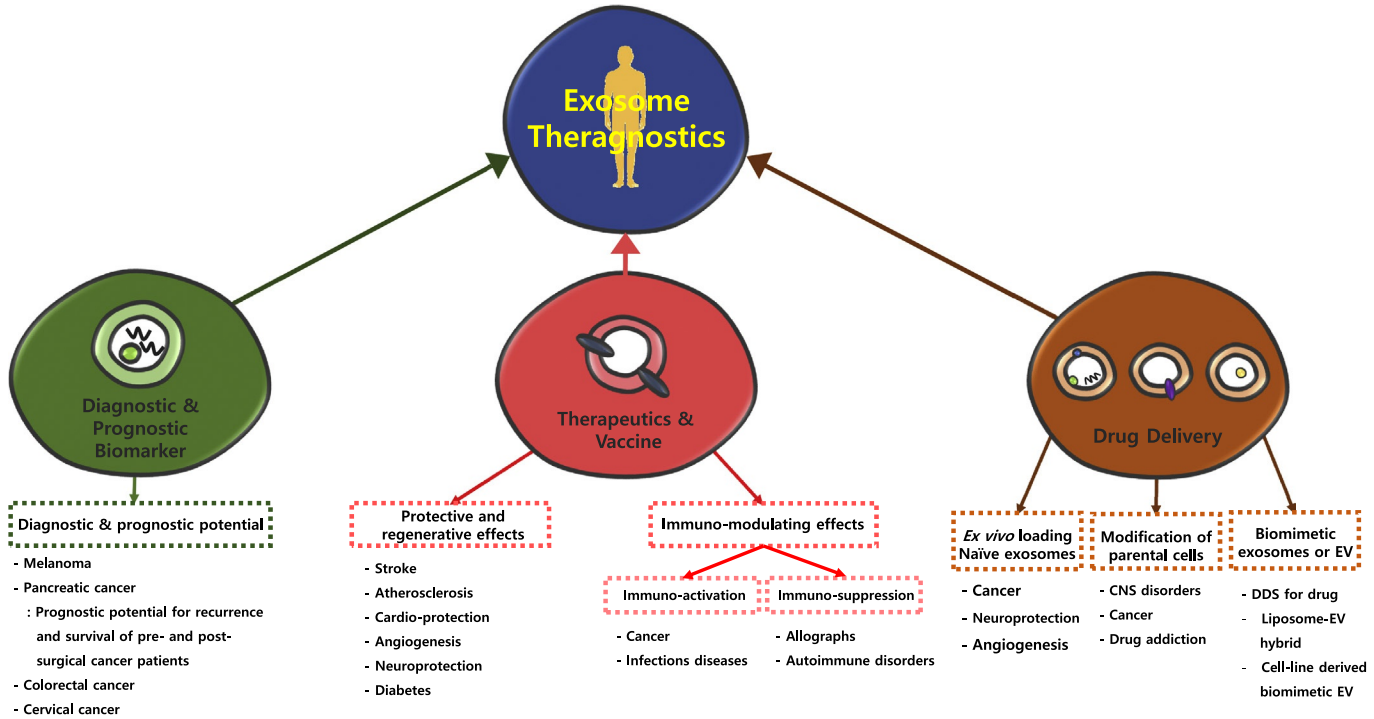
**TABLE 1** Pros and cons of extracellular vesicles for therapeutic delivery (see [1, 24, 68, 83–87])

Pros (advantages)	Cons (disadvantages)
<ul style="list-style-type: none"> <li>- Nanoscale vesicles for biocompatibility and stability in body fluids.</li> <li>- Naturally derived low immunogenicity.</li> <li>- Stealth capacity against immune system.</li> <li>- Inherent target properties with reduced off-target effects.</li> <li>- Ability of guiding therapeutic cargo across biological barriers, especially BBB.</li> <li>- Capacity to be loaded with specific small molecules such as miRNA and drugs.</li> <li>- Unique composition allows direct membrane fusion with target cell, for efficient cell uptake.</li> <li>- Safe in clinical trials.</li> <li>- Low inherent toxicity.</li> </ul>	<ul style="list-style-type: none"> <li>- Secretion and uptake mechanism, composition, and biological functions are not yet understood.</li> <li>- Impact on the target cell is unknown.</li> <li>- Isolation techniques with high efficiency and robust yield are lacking.</li> <li>- Scalable production difficult—there are no optimal purification methods: large-scale production is expensive and challenging.</li> <li>- Efficient loading methods without damaging EV integrity are lacking.</li> <li>- In vivo data have been less studied, and in vivo tracking requires further studies.</li> <li>- Clinical studies on therapeutic delivery are lacking.</li> <li>- Vesicles with heterogeneous constituents can be immunogenic.</li> </ul>

which needs to be carefully evaluated for EV-based products (see [88]). Fig. 2 shows the overall scheme of different modalities for using exosome-based formulations (see [89]).

Also, the loading efficiency of therapeutics into EVs remains inadequate. The relatively tight and ordered lipid bilayer impedes efficient loading of drugs into EVs without reducing membrane integrity. Such damage might alter the immune-oriented characteristics of EVs and make them visible to the mononuclear phagocyte system (see [89]). Therefore, ideal loading methods should not only possess high loading efficiency but also preserve the structural integrity of EVs and the functional integrity of therapeutics (see [83]).

A growing area of interest is the application of diverse nanotechnology-based DDS such as liposomes, polymeric nanoparticles, dendrimers, and magnetic nanoparticles (see [90]). These delivery systems are being used to deliver various types of cargo including chemotherapeutics, anti-inflammatory drugs and miRNAs (see [91, 92]). The nano-sized diameters of these DDS facilitate delivery through the blood and lymphatic systems with effective drug-loading capacity (see [93]). Moreover, our understanding has progressed regarding the



**FIG. 2** Schematic representation of various strategies of exosome-based diagnostic and therapeutic approaches in translational and clinical medicine. EV, extracellular vesicle.

in vivo pharmacokinetic behaviors of EVs such as circulatory half-life, tissue distribution in vivo, cellular uptake, and intracellular fates. This understanding is crucial to the clarification of the biological functions of exosomes and practical application of exosome-based therapeutics. To define the pharmacokinetics of exosomes clearly, the first step includes the evaluation of tissue distribution in vivo, in other words the so-called biodistribution of exosomes. Several labeling methods with small lipophilic fluorescence dyes have been introduced and used for that purpose for in vivo tracking. Although the reliability of in vivo analysis would be decreased by the release of free dye from exosomes, this strategy is a useful approach to assess the localization of exosomes to tissues (see [94, 95]). PKH67, a lipophilic fluorescent dye, was used to label highly metastatic B16F10 murine melanoma cell-derived exosomes that accumulated in the lung, bone marrow, spleen, and liver, enhanced endothelial permeability in the lung, and facilitated tumor metastasis to the lung (see [96]). In addition to fluorescent dyes, lipophilic near-infrared dyes such as 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiD) and 1,1'-dioctadecyltetramethylindotricarbocyanine iodide (DiR) have been widely used for the imaging of exogenously administered exosomes. DiD-labeled MSC exosomes were well distributed to the spleen and liver after intravenous administration in normal mice (see [94]). On the other hand, in a mouse model of acute kidney injury, these exosomes accumulated in the kidney, as well as the spleen and liver, after intravenous injection. This finding may explain how the intravenous administration of MSC-derived exosomes stimulates the recovery from cisplatin-induced acute kidney injury in SCID mice (see [97]). In the study of in vivo behavior of DiR-labeled exosomes of various cell types such as B16F10 murine melanoma cells, C2C12 murine myoblast cells, bone marrow-derived DCs, and HEK293T human embryonic kidney cells, exosomes were mainly distributed in the liver, spleen, lung, and gastrointestinal tract after intravenous administration. Among these exosomes, B16F10 murine melanoma cell-derived exosomes mainly accumulated in the lung compared with the exosomes derived from the other two types of murine cells. The highest accumulation in the spleen and liver was observed with DC exosomes and C2C12 exosomes, respectively. HEK293T exosomes mainly accumulated in the liver after intravenous injection and the liver, pancreas, or gastrointestinal tract after intraperitoneal or subcutaneous injection, respectively (see [98]). Because of its higher sensitivity and stability, the labeling of exosomes with radiotracer is a more appropriate method for quantitative assessment of the pharmacokinetics and tissue distribution of exosomes compared with labeling with fluorescence dyes or chemiluminescent proteins. For example,  $^{111}\text{In}$ -labeled PC3 exosomes rapidly disappeared from blood circulation and were primarily distributed in the liver (12% injection dose [ID]/g at 24 h) after intravenous injection (see [99]). Various types of cells recognize and take up exosomes. Therefore, identifying these cells is important for further exploration of the biology of exosomes and for the development of exosome-based therapeutics. The mouse DC-derived exosomes

were picked up by macrophages in the spleen and liver (see [90]), and exosomes derived from MDA-MB-231 breast cancer cells were taken up by macrophages in the lung and brain after intravenous administration, respectively. Exosomes derived from C2C12 cells, NIH3T3 cells, MAEC cells, and RAW264.7 cells were mainly picked up by macrophages in the liver after intravenous administration (see [100]). These results suggest that macrophages are the main cells that actively take up exogenous exosomes (see [101]). It has been predicted that exosomes are taken up by cells through the recognition of surface molecules on the membranes of the exosomes. Several studies investigated the molecules that may contribute to the *in vivo* pharmacokinetics of exosomes. An *in vitro* study proved that carbohydrate moieties on the membranes of exosomes contributed to the cellular uptake of exosomes (see [102]). Exosomes derived from tumor cells that metastasized to the lung (MDA-MB-231 and 4175) or to the liver (BxPC-3 and HPAF-II) mainly gathered in the lung and liver, respectively. A proteomic analysis of exosomes showed high expression of integrins  $\alpha 6\beta 4$  and  $\alpha V\beta 5$ , respectively. Exosomes collected from integrin  $\beta 4$ -knocked down 4175 cells showed reduced accumulation in the lung. These results demonstrate that integrins play a key role in the pharmacokinetics and tissue distribution of exosomes *in vivo* (see [101]). Exosomes derived from genetically modified immature DCs expressing the iRGD peptide showed a selective distribution to  $\alpha v$  integrin-positive tumor tissues (see [103]).

In addition to delivery of small RNA molecules, which depend on intracellular delivery to perform intrinsic functions, EVs have also been applied to deliver chemotherapeutic agents with the aim to increase their efficacy and reduce adverse effects. One study involved the use of exosomes to deliver curcumin to treat an inflammatory disease (see [104]). Exosomes are applied to form a complex with curcumin to enhance curcumin anticancer activity (see [105]). The intravenous injection of integrin-targeted, dendritic cell-derived EVs with the chemotherapeutic doxorubicin led to significant repression of tumor growth compared to free doxorubicin in a mouse model of breast tumor. Moreover, doxorubicin when loaded into EVs was shown to cause less cardiac damage, which is otherwise its most important dose-limiting adverse effect (see [103]). The advantage of exosomal doxorubicin versus liposomal doxorubicin involves the natural orientation of exosomal membrane proteins and their ability to interact with the receptors in the target cell plasma membrane (see [106, 107]). Furthermore, exosome-encapsulated paclitaxel compared with free taxol was shown to be more effective in controlling the growth of Lewis lung carcinoma metastases, and it holds significant potential for the delivery of various chemotherapeutics to treat drug-resistant cancers (see [108]). Repeated intraperitoneal injections of cisplatin-loaded EVs improved long-term survival of ovarian cancer-bearing mice as compared to free cisplatin, and intravenous injection of doxorubicin-loaded EVs delayed growth of established subcutaneous hepatic cancer (see [109]). Importantly, these exosome-encapsulated drugs did not adversely affect liver or kidney function, which is frequently observed after administration of free drugs.

Regrettably, the movement of large proteins through the blood-brain barrier (BBB) is severely limited. In fact, 98% of all potent drugs that may be better options for various CNS diseases are not applied in the clinic because they cannot cross the BBB (see [110]). Various nano-sized drug formulations have been developed to overcome this hurdle (see [111, 112]). Parkinson's disease (PD) is known to be associated with brain inflammation, microglia activation and secretory neurotoxic activities, including reactive oxygen species (ROS) (see [113, 114]). Samples of brain tissue from patients with PD have shown reduced levels of redox enzymes, catalase, and superoxide dismutase, and other antioxidants (see [115–117]), which indicates an impaired defense against oxidative stress and neurodegeneration in these patients. Among these molecules, catalase is one of the most effective natural antioxidants: it scavenges one million free radicals through a catalytic reaction. Therefore, successful delivery of catalase into the brain may be an important and possible approach to PD therapy (see [84]). EVs have been proposed as therapeutic delivery vehicles for the treatment of PD. Exosomes are readily taken up by neuronal cells *in vitro*, and a considerable number of exosomes was detected in PD mouse brain after intranasal administration. Catalase-loaded exosomes (ExoCAT) were shown to suppress microglial activation and protect neurons against ROS more efficiently compared to free catalase in *in vitro* and *in vivo* models of PD (see [84]). Therefore, ExoCAT is a more adaptable strategy for treating inflammatory and degenerative disorders such as PD (see [84, 118]). Although these reports are preliminary, the results have shown that exosomes are promising candidate drug delivery systems for the treatment of a variety of diseases.

Exosomes derived from human adipose tissue-derived mesenchymal stem cells (MSCs) are considered to have therapeutic value for treating AD (see [119]). Exosomes can be used *in vivo* as a vehicle to carry active neprilysin (NEP), the most important enzyme for  $\beta$ -amyloid ( $A\beta$ ) peptide degradation in the brain. MSC-derived exosomes also decrease intracellular and extracellular  $A\beta$  levels in the neuroblastoma cell line N2A *in vitro*. Therefore, human adipose tissue-derived MSC-originated exosomes are proposed as a potential therapy against AD from the point of view of their  $A\beta$ -degrading capacity. Recent studies have demonstrated that multipotent mesenchymal stromal cells (MSCs) hold great promise for neurovascular remodeling and neurological function recovery following a stroke. It has been demonstrated that MSC-derived exosomes have a neuroprotective effect against stroke due in part to changing the miRNA profile of exosomes during and after stroke (see [120]).

When we consider the problems associated with many current nanoparticulate delivery systems, exosomes as a mimic of “natural delivery systems” are a potential alternative for delivery of the biological molecules described above. Because of their small size and host-derived cellular product, these vesicles can avoid phagocytosis or degradation by macrophages and also circulate for long periods of time in the body. One of the interesting advantages of these delivery vehicles includes their ability to cross the BBB and arrive in the CNS

(see [121]). Insufficient knowledge of the nature of exosomes and their role in the pathophysiology of overall health and disease makes it complicated and difficult to predict long-term safety and therapeutic effects. In vivo trafficking of exosomes, their biological fate and their impact on targeted organs need to be understood with regard to therapeutic cargo loading and assembly for drug delivery (see [122]). Currently, there is no distinct optimal purification technique for isolation of exosomes with high purity (see [123]). The current isolation methods yield low quantities of exosomes, and their large-scale production for clinical studies and post drug approval is expensive and complicated (see [38]). It is highly likely that future clinical use will demand hybrid designs of exosomes (see [124]), and when combined with therapeutic cargo they may show undesirable effects. Even though extensive exosome biology is already known, exosomes comprise heterogeneous constituents and may show immunogenicity (stimulatory or suppressive) effects based on the nature of the donor cells. Exosomes provide immense promise and are a new therapeutic area for delivery of various synthetic and biological molecules in cellular therapy. Exosomes as drug delivery systems provide a major advantage as there is no undesired aggregation or homing of exosomes in the liver and/or first-pass effect before arriving in target sites. Well-characterized exosomes with long-term safety that deliver nucleic acids and therapeutic molecules between cells and through difficult-to-cross membranes such as BBB would have major practical significance. However, before these drug delivery systems become a therapeutic reality, components and processes including immune reactions need to be clarified (see [122]). The emerging evidence that tumor cell-derived exosomes have unique properties may be used to develop an exosome-based drug delivery system that is better than synthetic drug carriers. However, some limitations and hurdles must be overcome before exosome-based drug delivery systems can be used in the clinic. Important issues still need to be autologous or can non-immunogenic exosome factories. Therefore, a strategy needs to be developed for manufacturing vesicles for therapeutic application, with establishment of the producer cell type, physical methods to produce vesicular nanoparticles, enhancement of EV yield and scale-up, measurement of potency of EV-based products, and EV-inspired bioengineered artificial vesicles. Recently, a group of researchers developed a method to produce exosome-mimetic vesicles, which can overcome natural exosome limitations as like low drug-loading efficiency and low exosome production yields (see [125]). These chemotherapeutic-loaded nanovesicles, which are 100–200 nm in diameter, were generated by breaking down cells by serial extrusion through filters with diminishing pore size (see [126]). It was further suggested that these nano-vesicles with exosome-mimetic properties can be used as a platform for RNAi transfer to the cell cytoplasm (see [127]). However, the high level of cholesterol, ganglioside, and sphingomyelin in exosomal membranes leads to a more rigid bilayer structure than that of their parent cells (see [128]), which suggests that their fusion with lipid-based particles requires rough conditions (see [129]), such as aggressive freeze-thaw

processes (see [130]). To avoid the need for such conditions, Yang et al. [131] designed a virus-mimetic fusogenic exosome platform to deliver membrane proteins to target cell membranes, involving integrated vascular stomatitis virus G protein, a viral fusogen (see [131]). Interestingly, these methods allow easy exosome modification by fusing exosomes derived from modified cells with liposomes inserted with peptides, antibodies or polyethylene glycol.

Although there are currently many challenges in the treatment of cancer and other refractory diseases, exosomes, including exosome-mimetic nanovesicles, are considered valid diagnostic biomarkers and potential therapeutic tools. Moreover, along with chemical, physical, cellular and genetic engineering techniques, many existing exosome modification strategies are promising for application in various clinical situations (Fig. 2).

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