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A novel platform for cancer therapy using extracellular vesicles☆

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ABSTRACT

Extracellular vesicles (EVs) are nanometer-sized membranous vesicles and are involved in cell-to-cell communication. EVs contain several types of functional molecules, such as proteins, mRNAs, and microRNAs (miRNAs). Over the past several years, EVs have emerged as potential tools for a drug delivery system (DDS) that can target organs or cells. EVs have a function of organ tropism and are naturally occurring from cells. Therefore, EVs have expected as naturally DDSs, which have the organ tropism and a low side effect. Actually, some reports showed that EVs delivered drugs to specific organ. However, despite observed the organ tropism, the mechanisms of organ tropism of EVs are still unclear. Moreover, preservation and efficient collection of EVs are desired to be investigated. Here, we provide an overview of the methods for using EVs as DDSs.

Contents

1. Introduction

Effective therapeutic agents have been widely developed, if would be delivered the effective therapeutic agents to the target organ. However, it is a problem that non-targeted drugs are inefficient and have side effects when they are delivered systemically. The purpose of a drug delivery system (DDS) is to deliver a drug efficiently, improve the effect of the drug, and reduce its side effects [1]. The conventional DDSs, such as PEG [2–4] and liposome [5], are useful in drug delivery. However, despite the untiring efforts of researchers, the delivery to specific organ and the side effect of DDS remain unsolved problems.

DDSs are desirable for use in cancer therapy, and extracellular vesicles (EVs) have a potential to serve as natural DDSs for cells [6–9]. EVs are formed via the endosomal system, thus resulting in multivesicular bodies (MVBs) [10,11]. EVs are membranous vesicles of endocytic origin

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that are released by a variety of cell types into the extracellular space. Another population of EVs, shedding vesicles, exosomes, tolerosomes, and prostasomes, are formed directly through the shedding of the cellular membrane. However, there is still confusion regarding the terminology used to describe these secreted vesicles because it is difficult to distinguish the different types of vesicles after their secretion. To avoid confusion in the present article, we use the term "extracellular vesicles (EVs)" as a hypernym for all types of vesicles that are present in the extracellular space [12]. Importantly, it has been observed that EVs have a tropism to some organs or cells. Over the past several years, EVs have emerged as potential tools for DDSs to target organs or cells, and promising results have been achieved by using EV-based DDSs. Because of their biological significance, EVs have advantages for use as DDSs. Furthermore, EV-based DDSs are expected as a low-side-effect DDS, because EVs are naturally occurring from cells. Therefore, EV-mediated tumor-selective drug delivery or EV-mediated organ-selective drug delivery makes EVs an attractive DDS candidate. On the other hand, it is not matured that the methods of encapsulation of drugs, addition of organ tropism, preservation, and efficient collection of EVs.

In this review, we summarize the techniques for using EVs as DDSs. We also discuss the hurdles to be overcome to further develop EV-DDSs.

2. Techniques

2.1. Encapsulation of drugs to EVs

Drugs should be attached to or encapsulated in EVs for the vesicles to be used as DDSs. In previous studies, microRNA (miRNA) or existing drugs have been transfected or attached directly to EVs, fusion protein vector has been transfected to donor cells, and methods for encapsulating existing drugs in EVs using a discharge mechanism have been attempted (Table 1).

Human monocytic leukemia THP-1 cells have been used as donor cells [13]. EVs were collected from THP-1 cells, which transfected chemically modified microRNA (miR)-143 with an aromatic 3′-benzene-pyridine analog added to the 3′-overhang region of the RNA strand. The miR-143 levels were significantly increased in the serum, tumor, and kidneys of host animals that were injected with EVs. It has also been reported that miR-130b could be overexpressed in EVs from HeLa-229 cells by transfection of the vector [14]. In another study, argonaute 2 (Ago2) and heat shock protein 90a (HSP90a) were enriched in miR-130b-packaging EVs. These proteins protect against degradation. Furthermore, Lattanzi *et al.* reported that a mutant of the HIV-1 Nef protein, Nefmut, was efficiently incorporated with foreign proteins in EVs when fused with the proteins at its C-terminus [15]. Nefmut-fused foreign protein expression vector was transfected to 293T cells. Furthermore, the authors showed that vesicular stomatitis virus G protein (VSV-G) fusion Nefmut EVs have potential as an EV-based vaccine.

Some groups have demonstrated that paclitaxel or doxorubicin binds with EVs when they are incubated at 37 °C [16,17]. Human U937 monocytic cells and mouse Raw264.7 macrophages have been used as donor cells of EVs [17]. In that study, collected EVs were incubated with 400 μg/mL doxorubicin for 2 hrs at 37 °C and were then injected intravenously into mice. This report showed that doxorubicin was incorporated into the EVs and that these EVs accumulated in mouse colon adenocarcinoma CT26 cells *in vivo*. However, the EVs also accumulated in the liver. In other reports, curcumin or an activator of transcription 3 (Stat3) inhibitor, JSI124, was mixed with mouse lymphoma cell-line EL-4-derived EVs at 22 °C and bound to the EVs [18,19]. EVs have also been collected from a mouse brain endothelial cell line by curcumin treatment (7.5 μM) for 72 hrs [20]. One study investigated four different methods for incorporating catalase into EVs from a Raw 264.7 macrophage cell line [21]. Method I involved incubation at room temperature (RT) for 18 hrs with or without saponin; Method II involved freeze–thaw cycles that were repeated three times at −80 °C and at room temperature; Method III was sonication (500 v, 2 kHz, 20% power, 6 cycles by 4 s pulse/2 s pause); and Method IV was extrusion, in which a catalase mixture with EVs was extruded (10 times) through an avanti lipids extruder with a 200-nm-diameter pore. The authors reported that sonication was the result of the high loading efficiency and high stability of catalase. Electroporation has been used to load doxorubicin [22] and small interfering RNA (siRNA) [23] into EVs.

Interestingly, it has been reported that melanoma cells treated with the anticancer drug cisplatin eliminated the cisplatin through EVs [24]. Secreted EVs were found to contain cisplatin, and the cisplatin concentration was higher in an acidic environment because EV secretion from cells increased the pH levels. Additionally, Pascucci *et al.* suggested that mesenchymal stromal cells (MSCs) could be used as drug packaging cells for EVs [25]. SR4987 established from bone marrow cells of BDF/1 mice was cultured for 24 hrs in a culture medium containing 2000 ng/mL of paclitaxel. After 48 hrs, the conditioned medium was

Table 1
The methods of encapsulation of drugs.

Drugs		Donor Cell of EVs	Methods	References
miRNAs	miR-143 (chemical modified)	THP-1 (human monocytic leukemia cell)	Transfection of expression vector	[13]
	miR-130b	HeLa-229	Transfection of expression vector	[14]
siRNA	BACE1 siRNA	Primary dendritic cells harvested from murine bone marrow	Electroporation (encapsulated)	[23]
Drugs	Vesicular stomatitis virus G protein (VSV-G)	HEK293T	Transfection of HIV-1 Nef protein-fused foreign protein expression vector	[15]
	Paclitaxel, doxorubicin	U-87 MG (human brain neuronal glioblastoma–astrocytoma) bEND.3 (brain endothelial cell)	Cells were incubatied at 37 °C, 2 hrs with drugs. (incorporate in EVs)	[16]
	Doxorubicin	U937 (human monocytic cell) Raw264.7 (mouse macrophage)	Cells were incubatied at 37 °C, 2hrs with drugs. (incorporate in EVs)	[17]
	JSI-124 (an activator of transcription 3 inhibitor)	EL-4 (mouse lymphoma cell)	Cells were incubated at 22°C with drugs. (bind to EVs)	[18,19]
	Curcumin	Mouse brain endothelial cell	Cells were treatment with drug for 72 hrs.	[20]
	Catalase	Raw264.7	(1) Incubation at room temperature for 18 hrs with or without saponin (2) freeze–thaw cycle (three times at −80°C and room temperature) (3) sonication (4) extrusion	[21]
	Doxorubicin	Mouse immature dendritic cells	Electroporation	[22]
	Cisplatin	Me30966 (human metastatic melanoma)	Cells were treatment with drug in acidic pH condition.	[24]
	Paclitaxel	SR4987 (established from BDF/1 mice from bone marrow cells)	Cells were treatment with drug for 24 hrs.	[25]

Table 2
Organ tropism by EVs component.

Component on EVs	Tropism	References
iRGD peptide (CRGDKGPDC) * Transfection of Lamp2b-fused foreign protein expression vector	Tumor (αV-integrin)	[22]
Rabies viral glycoprotein (RVG)	Brain	[23,26]
Folate receptor-α	Brain (astrocytes, neurons)	[27]
M2 macrophage (differentiated from Raw 264.7 cells)	Homing to inflamed brain tissues	[28]
Tspan8	Rat aorta endothelial cells Rat lymph node stroma cells Bone marrow cells Lymph node cells $CD54^+$ leukocytes	[29,30]
Tspan8-β4	Rat lung fibroblasts Rat lymph node stroma cells Lymph node cells $CD54^+$ leukocytes Peritoneal exudate cells Kidneys Pancreas	[29,30]
Tspan8 containing the N-terminal region of CD9	Rat aorta endothelial cells Rat lymph node stroma cells $CD44^+$ leukocytes	[29,30]
Unmodified EVs from rat pancreatic adenocarcinoma cell line (BSp73AS)	Rat lung fibroblasts Rat lymph node stroma cells	[29,30]

collected, and EVs were purified by ultracentrifugation. The concentration of paclitaxel in EVs was 11.68 ng/mg per EV protein. Importantly, when human pancreatic adenocarcinoma CFPAC-1 cells were treated with EVs containing paclitaxel, EVs that contained paclitaxel showed antitumor activity.

2.2. The organ tropism of EVs

Tumor tropism is important for the use of EVs as tumor therapies (Table 2). In this section, the techniques of organ tropism of EVs are summarized.

One study reported that an integrin-specific iRGD peptide (CRGDKGPDC) is able to bind to αV-integrin [22]. In that experiment, the authors used an MDA-MB-231 tumor treatment. The iRGD peptide that fused with a membrane protein (Lamp2b) in EVs had the potential to deliver treatment specifically to a tumor *in vitro* and *in vivo*. Several reports have given EVs tropism to the brain through the transfection of surface proteins to donor cells. Alvarez-Erviti *et al.* established a delivery system to mouse brains [23,26]. Self-derived dendritic cells (DCs) were used as EV donors because of their effects on reducing immunogenicity, one of the reasons that EVs are a candidate for a low side effect DDS. DCs were transfected with expression vector containing a Lamp2b gene and a neuron-specific rabies viral glycoprotein (RVG) peptide gene, and secreted EVs were corrected from the supernatant. To use the siRNA delivery system, BACE1 siRNA was included using an electroporation method. BACE1 is important for Alzheimer's disease pathogenesis. These Lamp2b-RVG-expressed and BACE1-siRNA-containing EVs were injected intravenously. Importantly, these EVs downregulated BACE1 protein in cortical tissue *in vivo*. A pre-experiment using GAPDH siRNA significantly downregulated GAPDH protein in the striatum, midbrain, and cortex. Furthermore, folate receptor-α (FRα) was reported to be one of the molecules responsible for brain parenchyma tropism [27]. Rat choroid plexus Z310 cells were transfected with FRα expression vector. Then, FRα was enriched in the EV fraction. When intraventricularly injected into mice, FRα-positive EVs might initially be taken up by astrocytes and then delivered to neurons. The authors argue that this function might be a transport medium for micronutrients. A glial cell-line derived neurotropic factor (GDNF)-overexpressing Raw 264.7 macrophage was used in the treatment of Parkinson's disease in a 6-hydroxydopamine-induced Parkinson's disease model [28]. EVs from a GDNF-overexpressing Raw 264.7 macrophage contain a high amount of GDNF, which can promote the regeneration of neurodegenerative disorder neurons and protect these cells from toxic injuries. Raw 264.7 macrophages were differentiated to an M2 regenerative subtype. Importantly, systemically injected GDNF-overexpressing M2 macrophages were homed to and mediated neuroprotection in brain tissues. The authors suggested that drug-secreting organ homing cells are useful for drug delivery and can be used as carriers for delivery of anticancer drugs to the brain.

Other studies have attempted to instill organ tropism. Rana *et al.* reported that tetraspanin EVs contribute to target cell selection [29,30]. The rat pancreatic adenocarcinoma line BSp73AS (AS) was used as a donor cell and was transfected with Tspan8, Tspan8-β4, or AS-Tspan8/CD9n (Tspan8 containing the N-terminal region of CD9) expression vector. These tetraspanin-overexpressing cells were cultured and EVs were collected from the supernatant. The EV uptake differed depending on the EV-recipient cell. EVs containing Tspan8 and Tspan8/CD9n preferentially bound to rat aorta endothelial cells and rat lymph node stroma cells (LnStr); unmodified EVs and EVs containing Tspan8-β4 bound preferentially to rat lung fibroblasts (Fb) and LnStr. EVs containing Tspan8 bound preferentially to bone marrow cells and lymph node cells. EVs containing Tspan8-β4 bound to lymph node cells. As an example of the dependency of the recipient cell, EVs Tspan8/CD9n were preferentially taken up by $CD44^+$ leukocytes, whereas EVs containing Tspan8 and Tspan8-β4 were mostly incorporated into $CD54^+$ cells. Of note, EVs containing Tspan8-β4 bound efficiently to peritoneal exudate cells, kidneys, and the pancreas *in vivo* [29].

Direct injection is an effective method of EVs delivery, not systemically injection. EVs containing prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP), which are tumor-associated antigens, were used in prostate cancer treatment by subcutaneous injection [31]. EVs containing tumor-associated antigen were increased the PSA and PAP IgG2a/IgG1 ratio, which is Th1-biased immune response, suggesting that this therapy induced against the tumor antigen in each model. Another group reported that EVs binding curcumin inhibited LPS-induced brain inflammation. Intranasally injected EVs were taken up in the brains of mice through microglial cells [18]. In this study, EVs from EL-4 were taken up by both microglial cells (approximately 60%) and non-microglial cells (approximately 40%). Furthermore, Stat3 inhibitor JSI-124-binding EVs inhibited glioblastoma GL26 growth [18].

2.3. Techniques used for collection, measurement of concentration, and preservation of EVs

Because EVs are heterogeneous, a specific population of EVs must be collected to be used appropriately. Moreover, the methods of efficient collection of EVs are desired. Previous reports have mainly used the ultracentrifugation method [22,26,32–35] and FACS methods [36,37] for collecting EVs. However, bulk EVs were collected using these methods. Nordin *et al.* compared EVs that were collected using ultrafiltration with those collected using ultracentrifugation [38]. Importantly, it was reported that the *in vivo* biodistribution of EVs was different between the methods. These observations suggested that bulk EVs are heterogeneous populations and that there is a need to collect a specific population of interest.

EV concentration is also an important factor in DDS use. The existing drug concentration or miRNA can be measured with a fluorescence spectrophotometer or through real-time PCR. In another study, Rupert *et al.* used label-free, surface-based sensing with surface plasmon resonance (SPR) to determine the concentration of EVs in solution [39]. Accurate and standardized quantification of such EVs was also required. Maas *et al.* compared different methods for determining EV concentration, including nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (tRPS), and high-resolution flow cytometry (hFC) [40]. The measurable concentration range of NTA was 9.0×10^7

particles/mL to 2.9×10^9 particles/mL. In contrast, the tRPS was different between the particle size and concentration (203 nm beads were analyzed at 9.1×10^7 particles/mL to 2.9×10^9 particles/mL, whereas 115 nm particles were analyzed at 3.6×10^8 particles/mL to 2.3×10^{10} particles/mL). However, hFC allows for accurate quantification at lower particle concentrations (4.6×10^6 particles/mL to 7.3×10^7 particles/mL). The concentration of EVs can be accurately measured using each of the different methods, although the authors suggested that (1) particles with low fluorescence intensity may not be detected and (2) the removal of unbound fluorescent dye is important when using the hFC technique.

A previous report studied appropriate methods of EV preservation, comparing urinary EVs collected and preserved at 4°C, −20°C, and −80°C [41]. An appropriate method for preserving EVs for a DDS has not been established.

3. Cancer therapy

3.1. Primary tumor therapy

iRGD-Exos-doxorubicin suppressed breast tumor growth in an MDA-MB-231 tumor-bearing nude mouse model [22]. EVs were injected into mice intravenously and accumulated in tumor tissue. This result suggested that EVs containing doxorubicin inhibited tumor growth.

Interestingly, Yang *et al.* performed drug delivery to the brain using a zebrafish brain tumor model [16]. They reported that endothelial brain cell line bEND.3-derived EVs could accumulate in the brain and brain tumor tissue across the blood–brain barrier (BBB). In another study, curcumin-primed EVs from a mouse brain endothelial cell line were used to treat endothelial cell dysfunction during hyperhomocysteinemia *in vitro* [20]. Curcumin-primed EVs have the potential to alleviate endothelial dysfunction. Another study reported that miR-9 in mesenchymal stem cell–derived EVs became chemosensitive in glioblastoma multiforme cells [42].

3.2. Immunotherapy

Raposo *et al.* first reported that B lymphocyte–derived EVs induced antigen-specific MHC class II–restricted T-cell responses [43]. Since then, it has been reported that tumor-peptide pulsed DC–derived EVs have potential with respect to cancer immunotherapy because they suppress tumor growth [44]. Wolfers *et al.* reported that tumor-derived EVs containing a tumor antigen may have activated a tumor immune response [45]. Tumor-derived EVs were taken up with DCs and activated DCs induced $CD8^+$T-cell-dependent antitumor effects. Another study reported that Gp120 stimulates Gp120-specific CTL responses and then long-term cancer immunity against Gp120-expressing B16 melanoma *in vivo* [46]. It has also been reported that EVs from the rat pancreatic adenocarcinoma cell line BSp73ASML (ASML) have potential use as an adjuvant therapy in immunotherapy [47]. EVs from ovalbumin (OVA)-pulsed DCs induced efficient $CD4^+$ Th cell-independent $CD8^+$ CTL responses *in vivo* [46,48,49]. Furthermore, it has been suggested that vaccination with a tumor antigen containing EVs activated an antitumor response against OVA-transfected BL6-10 melanoma cells. An EV vaccine derived from colorectal cancer has also been reported. In that study, EVs from NB4 cells, a human acute promyelocytic leukemia cell line, activated CTLs through DC activation [50].

3.3. Clinical trials

Clinical trials using EVs have been reported, with several types of clinical trials undertaken to investigate cancer therapy [51]. EVs from plants containing curcumin are used in colorectal cancer therapy. However, these EVs were found to deliver curcumin to both healthy and malignant colon tissues. EVs from grapes are used to prevent oral mucositis associated with chemoradiation in cases of head and neck cancer. Furthermore, it has been reported that EVs from DCs have been used to deliver a vaccine as part of immunotherapy in cases of unresectable non-small-cell lung cancer [52]. In another study, EVs from autologous DCs were used in the vaccination of metastatic melanoma patients [53]. It has also been reported that EVs from ascites were used for immunotherapy in colorectal cancer in combination with granulocyte–macrophage colony-stimulating factor (GM-CSF) [54]. However, Gundogan *et al.* suggested that clinical translation is limited by the lack of appropriate, scalable, and both cost- and time-effective nanotechnologies for the purification and loading of EVs [51,55].

On the other hand, a side effect is one of the important problem as DDS. However, it has never discussed about side effects. Dommelen *et al.* proposed that proteomic analysis of EVs is important, because it prevents from potential unwanted side effects, such as “spread of oncogenes, prions, inflammatory cytokines or viral particles” [55].

4. The biodistribution of EVs

Many researchers had observed the tropism of EVs *in vivo*. On the other hand, although EVs have unique proteins, researchers have not elucidated the mechanisms of clearance and biodistribution of unmodified tumor-derived EVs. Several groups have demonstrated that primarily unmodified tumor-derived EVs were incorporated into the liver [17,32,33,56].

One report on EV labeling observed the biodistribution of EVs containing gaussia luciferase (gLuc)-lactadherin, [33]. A gLuc-lactadherin fusion protein expression vector was transfected to the B16–BL6 murine melanoma cell line. Importantly, the results indicated that lactadherin has a tendency to concentrate in EVs; its level was approximately 10,000 times higher compared with the vector that did not contain lactadherin protein. In that study, EVs containing gLuc-lactadherin were injected intravenously (i.v.) and distributed mainly to the liver and then the lungs. In contrast, EVs from human embryonic kidney (HEK) 293T cells were observed to have the highest signal in the spleen; in that case, EV signal was also high in the liver, lungs, and kidneys [57]. HEK293T cells were transfected with a gaussia luciferase gene, and EVs were then collected from the supernatant using ultracentrifugation. These EVs were injected into mice, and their biodistribution was observed. Importantly, lower signals were observed in the brain, heart, and muscle. Another report has shown that the EV biodistribution was different when using two different collection methods [38]. EVs collected by ultracentrifugation accumulated mainly in the liver, but the accumulation of EVs was also observed in the lungs, spleen, and kidneys. Conversely, EVs collected by ultrafiltration with subsequent liquid chromatography accumulated in the lungs to a slightly lesser extent than did the EVs collected by ultracentrifugation. EVs collected by ultrafiltration with subsequent liquid chromatography also accumulated mainly in the liver. The biodistribution of EVs from several types of cells has also been observed [58]. HEK293T-derived EVs were injected into mice intravenously. EVs accumulated mainly in the liver, spleen, gastrointestinal tract, and lungs. It was observed that EVs were accumulated dose-dependently in each organ. The biodistribution of EVs from a muscle cell line; C2C12, a melanoma cell line; B16F10; and primary immature bone marrow–derived DCs were also observed in mice. The biodistribution of EVs from these cells were mostly consistent with the biodistribution of HEK293T-derived EVs. However, it has been reported that HEK293T-derived EVs were accumulated in tumor tissue and that the EV accumulation was 3% of the total tissue fluorescence *in vivo*. Furthermore, it was observed that EVs from mesenchymal stem cells accumulated in the kidneys of mice with acute kidney injury [59]. However, the accumulation of EVs was mainly observed in the liver and spleen. EVs containing RVG from DCs accumulated in the brain, heart, and muscle, compared with EVs that did not contain RVG. Those

EVs containing RVG were modified because RVG has an affinity to the nicotinic acetylcholine receptor.

Intriguingly, it has also been observed that EVs have a tropism to bone marrow [49,50], the lungs [33,57,60–62], brain [57,62–64], lymph nodes [64,65], and tumors [17]. Intratumoral injection of EVs allowed for more efficient uptake by tumor cells compared with liposomes [56]. However, no report has clearly determined the mechanism responsible for organ tropism in EVs. Importantly, Zomer *et al.* reported that tumor cell-derived EVs were directly taken up by tumor cells using the Cre-LoxP system *in vivo* [66]. Cre recombinase (Cre)-expressing MDA-MB-231 breast cancer cells secrete Cre-containing EVs. When Cre-containing EVs incorporated by reporter cells and Cre in EVs had enzyme activity, reporter cells changed reporter gene expression from DsRed to eGFP. Surprisingly, reporter cancer cells incorporated Cre-containing EVs both by direct injection and co-transplant with Cre-expression cancer cells *in vivo*. Furthermore, Cre-containing EVs were incorporated by reporter cancer cells from orthotropic transplanted Cre-expressing cancer cells to opposite site transplanted reporter cancer cells *in vivo*. This methods can be used to elucidate the transport and organ tropism mechanisms of EVs *in vivo*.

As described in Section 2.2, the tropism of EVs is provided by modification of EVs-surface proteins. In this section, we discussed the biodistribution of EVs, which is observed many researchers. These tropism mechanisms of EVs have not been uncovered; however, it is possible that the mechanisms of EVs tropism can apply to DDSs.

5. Future perspectives

Through the untiring efforts of researchers, EVs are being explored as potent therapeutics because of their efficient transfer of proteins, mRNA, miRNA, and existing drugs in selective targets. To apply EVs as a DDS, it is important to (1) attach or encapsulate a “drug” to EVs, (2) preserve the EVs, and (3) establish organ tropism. Some methods for encapsulating drugs to EVs have been developed; oligonucleotide-based drugs are especially useful when they are transfected to EVs because oligonucleotides, such as miRNA, are a major component of EVs. EVs seem to be enclosed oligonucleotide-based drugs by using transfection methods to donor cells. In contrast, no suitable methods of preserving EVs as a DDS have been reported. Furthermore, no study has clearly targeted intact EVs to cancer cells or target organs. As mentioned previously, with respect to EV biodistribution, most EVs accumulate in the liver *in vivo*. Although some reports have shown that EVs accumulate in the bone marrow, lungs, brain, lymph nodes, and tumor tissue, these reports have not provided sufficient evidence supporting specific organ tropism. Therefore, previous reports have generally used bulk EVs, and not those purified with EV markers. It has been reported that EVs containing RVG have a tropism to the brain. This result suggests that the modification of EV membrane proteins provides organ tropism. For these reasons, it is important to find the proteins that are responsible for natural organ tropism in EVs. Moreover, despite the general understanding that EVs derived from cells have this organ tropism, there is no quantitative or qualitative analysis on the classification of EVs. Further studies are needed for the proper application of EVs as a DDS, with a particular focus on the mechanisms responsible for organ tropism in EVs.

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