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Exploring interactions between extracellular vesicles and cells for innovative drug delivery system design

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ABSTRACT

Extracellular vesicles (EVs) are submicron cell-secreted structures containing proteins, nucleic acids and lipids. EVs can functionally transfer these cargoes from one cell to another to modulate physiological and pathological processes. Due to their presumed biocompatibility and capacity to circumvent canonical delivery barriers encountered by synthetic drug delivery systems, EVs have attracted considerable interest as drug delivery vehicles. However, it is unclear which mechanisms and molecules orchestrate EV-mediated cargo delivery to recipient cells. Here, we review how EV properties have been exploited to improve the efficacy of small molecule drugs. Furthermore, we explore which EV surface molecules could be directly or indirectly involved in EV-mediated cargo transfer to recipient cells and discuss the cellular reporter systems with which such transfer can be studied. Finally, we elaborate on currently identified cellular processes involved in EV cargo delivery. Through these topics, we provide insights in critical effectors in the EV-cell interface which may be exploited in nature-inspired drug delivery strategies.

Contents

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1. Introduction

Intercellular communication can occur via a wide variety of signaling processes and is indispensable for the maintenance of homeostasis. One of these processes is the cellular secretion and uptake of extracellular vesicles (EVs), which takes place in virtually all cells in the body. These lipid bilayer-enclosed vesicles can originate from specific types of endosomes – so called multivesicular bodies (MVBs) – which can fuse with the plasma membrane to release their content in the extracellular space. Alternatively, EVs can be formed by budding and fission from the plasma membrane [1]. EVs derived from MVBs are generally referred to as exosomes, whereas EVs derived from the plasma membrane are often termed microvesicles, ectosomes or microparticles, depending on the cell type secreting the vesicles. Due to their endosomal origin, exosomes can span sizes of 50 nm to up to 200 nm, whereas plasma membrane-derived EVs can be sized between 50 and 1000 nm [2]. Over the past two decades an increasing number of functionalities has been ascribed to EVs, attracting the attention of academic and industrial researchers in a variety of research fields. EVs were initially believed to serve as cellular waste disposal systems. For example, reticulocytes were found to encapsulate the transferrin receptor [3] and other redundant cellular components in EVs during their maturation to erythrocytes, after which these EVs are effectively cleared by phagocytes [4]. In a similar fashion, cancer cells secrete chemotherapeutic agents in EVs to reduce cellular drug accumulation and avoid cell death [5]. Hence, EV secretion is an important mechanism by which cells shuttle harmful substances and waste products towards clearance pathways. Later discoveries have led to the consensus that EV release and uptake is a highly conserved process between different cell types and organisms. Moreover, numerous functionalities besides waste disposal have been ascribed to EVs, both in physiological and pathological contexts. For example, EVs can exert immunostimulatory functions by presenting antigens on MHC complexes and costimulatory molecules to T cells [6], can contribute to neurotoxicity in Alzheimer's disease by the transfer of amyloid-β from astrocytes to cortical neurons [7,8], can act as long-distance effectors of stem cells in various tissue regeneration processes [9], and play roles in the onset of cancer metastasis [10] and modulation of the tumor microenvironment to facilitate cancer progression [11,12]. These functionalities are orchestrated by an interplay of the biological cargo of EVs and their surface molecules. EVs contain a range of cellular proteins, lipids and coding and non-coding nucleic acids, including miRNAs and other small RNA species [13], mRNAs [14] and even DNA [15]. This content is highly dynamic and can change based on the state and origin of the EV-secreting cell [16]. As a consequence, EVs are typically very heterogeneous in both content and functionality [17–19]. The native ability of EVs to encapsulate content of the EV-producing cell, transport this content through biological fluids and functionally deliver it to other cells has sparked the incentive to exploit EVs for diagnostic and therapeutic purposes [20]. This interest has not remained restricted to academia but has also fueled the establishment of several successful EV-focused companies. Furthermore, clinical trials with EV-based therapies have already started and have yielded promising results [21].

Despite these ongoing efforts to utilize EVs in therapeutic and diagnostic platforms, our knowledge on the mechanisms by which EVs can transfer content to recipient cells has remained limited. Such knowledge is of vital importance for the further development of EV-inspired therapies. For example, targeted inhibition of specific proteins on tumor-derived EVs may abolish tumor growth and/or promote recognition of tumor cells by the immune system [22,23]. In addition, the ability of EVs to transfer biologically active cargo to specific recipient cells may be restricted to specific subpopulations [24], which may be enriched for therapeutic applications. Alternatively, incorporation of known functional EV components in synthetic drug delivery systems could greatly promote their efficacy and tolerability [25].

In this review, we explore the critical mediators in the EV-recipient cell interface that could govern functional EV content transfer. First, we will sketch the current landscape of EV applications in drug delivery and highlight the importance of the EV surface content in such applications. Then, we will zoom in on important molecules on the surface of EVs which could directly or indirectly contribute to their ability to functionally transfer their content to recipient cells. In addition, we will review the various cellular reporter systems used to study functional EV cargo transfer. Finally, we will elaborate on the lessons learnt from such reporter systems regarding the cellular processes and proteins that may govern functional EV uptake. Together, these topics can provide valuable leads for further research into EV biology and their applications in therapy.

2. The appeal of EVs as drug delivery systems

One hallmark of EVs is that they are - in theory - biocompatible. They are continuously secreted into our bodily fluids as part of an intercellular communication network and should be capable of circumventing immune recognition and clearance issues encountered by most synthetic drug delivery systems (e.g. liposomes, lipid nanoparticles and polymers [26]) in order to exert their functions. Also exogenously isolated and intravenously administered EVs may display biocompatible features [27]. A recent study performed by Saleh *et al.* showed that EVs derived from Expi293F cells (an engineered HEK293 cell line optimized for high-density cultures and protein production) were well tolerated in immunocompetent BALB/c mice and did not increase serum levels of liver transami-

nases and inflammatory cytokines. Moreover, no histological abnormalities were observed in spleen, liver and kidneys of the mice [28]. Additionally, in a vaccination approach, EVs derived from serum from pigs infected with a Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) were well tolerated after intramuscular injection in pigs [29]. As pigs are known to easily develop hypersensitivity reactions upon infusion of liposomes [30], these results suggest that EVs can be better tolerated than conventional drug delivery systems. However, it should be noted that EVs were administered at markedly lower doses than liposomes in these studies. In another study by Mendt *et al*, human fibroblast-derived EVs did not alter liver or kidney function or blood chemistry after repeated administration to immunocompetent mice every 48 h for 4 months. In addition, no changes in the abundance of lymphocytes and myeloid cells were observed in the spleen, bone marrow or thymus after treatment with EVs derived from various human cell sources every 48 h for 3 weeks, whereas thymic $CD3^+$ T cell populations were slightly depleted after treatment with liposomes [31].

Importantly, the high tolerability of EVs observed in animal models may extend to humans as well. In the first phase I clinical trials on EVs, EVs were derived from autologous tumor antigen-pulsed dendritic cells and administered subcutaneously and intradermally four times with weekly intervals to late-stage melanoma or non-small cell lung cancer (NSCLC) patients as an anti-tumor vaccination strategy. The therapy was found to be well tolerated with only slight inflammatory reactions at injection sites [32,33]. Similar results were obtained when autologous malignant ascites-derived EVs were repeatedly subcutaneously administered to late-stage colorectal patients [34]. A follow-up phase II study in NSCLC patients revealed that autologous dendritic cell-derived EVs were also well tolerated upon sustained dosing (up to 27 injections) [35]. Moreover, in a phase II/III study no adverse effects were reported after two intravenous administrations of allogeneic human cord blood mesenchymal stem cell-derived EVs to patients with chronic kidney disease [36]. Taken together, these (pre)clinical studies suggest that EVs from various sources have a favorable safety profile for application in EV-based therapies.

The biocompatibility of EVs goes hand in hand with their surface composition. Although the exact molecules responsible for the frequently reported immune tolerance of EVs remain elusive, it is clear that EVs – depending on their cell source – can display molecules which can favor their interactions with specific cells or tissues, which can be beneficial in the context of targeted drug delivery. For example, a recent study showed that EVs derived from xenografted HT1080 tumors in mice accumulated in the same tumors after intravenous injection, whereas EVs derived from xenografted HeLa tumors accumulated in the HT1080 tumors to a lesser extent [37]. These data are in line with a landmark study from Hoshino *et al.*, which demonstrated that though integrin expression, EVs from various tumor cell lines exhibited preferential accumulation in brain, lung or liver tissues, facilitating the formation of a pre-metastatic niche for their parent cells [38]. Preferential uptake of "self-derived" EVs by established tumors and metastases was also reported for B16F10-derived EVs in immunocompetent mice [39]. In addition, M1-polarized macrophage-derived EVs showed increased accumulation in ovarian orthotopic tumors after intraperitoneal injection compared to liposomes [40], suggesting that EVs may show some degree of specificity for interaction with specific organs and/or cell types. However, despite this and other evidence for cell-specific tropism of EVs from various sources [38,41,42], it is clear that EV biodistribution is overall remarkably similar to that of synthetic nanoparticles, where organs of the reticuloendothelial system (i.e. liver, spleen and lungs) are the predominant sites of nanoparticle accumulation [43,44]. Moreover, other studies failed to show any preferential accumulation of tumor cell-derived EVs (including B16F10-derived EVs) in their respective tumors after intravenous administration [45,46], indicating that the presumed organ tropism of EVs is likely subtle and heavily influenced by the applied EV isolation protocol, or may be only relevant in the context of cell-specific uptake and retention *within* organs.

Another intriguing aspect of EVs is their potential ability to cross biological barriers such as the blood brain barrier, which is notoriously difficult to breach using synthetic drug delivery vehicles [47]. In a pioneering study, Alvarez-Erviti and coworkers showed that siRNA-loaded, targeting peptide modified immature dendritic cell-derived EVs could achieve knockdown of the *BACE1* gene (implicated in Alzheimer's disease) in specific areas of the brain after intravenous injection [48]. Furthermore, repeated administration of the EVs did not lower their efficacy, a phenomenon often observed for viral particles due to their recognition by the immune system [49]. Later studies showed that these EVs could also be exploited delivery of siRNA and siRNA-expressing minicircles to the brain in the context of Parkinson's disease [50,51]. Further evidence for the capacity of EVs to cross the blood brain barrier comes from a number of studies showing that brain-derived EVs can be detected in peripheral blood [52]. Despite these encouraging results, it should be noted that EV surface engineering with targeting ligand appears to be a prerequisite to achieve brain delivery after systemic administration [53].

Nevertheless, the theoretical benefits of EVs as next-generation biocompatible lipid-based drug carriers are appealing and have been extensively explored over the last decade.

3. EVs for the delivery of small molecules

As EVs have a similar structure (i.e. lipid bilayer membrane and aqueous core) as liposomes, they could offer the same advantages for the delivery of small molecules, while potentially avoiding some of the drawbacks of traditional lipid-based nanoparticles. For example, EVs may circumvent the accelerated blood clearance (ABC) effect, which often occurs after repeated administration of PEGylated nanoparticles, lowering their efficacy [54]. Furthermore, the proteinaceous membrane of EVs may extend the repertoire of drug molecules that can be stably incorporated beyond that explored for liposomal systems.

3.1. Curcumin as a model lipophilic drug molecule

In one of the first studies examining loading of small molecules in EVs, Zhang and colleagues reported that the anti-inflammatory and hydrophobic agent curcumin could be loaded into (presumably the membrane of) EVs by simple mixing, which greatly prolonged its circulation time and improved its ability to protect mice against LPS-induced septic shock [55]. This study was followed by others describing improved potency of curcumin after loading in EVs due to its improved bioavailability. For example, Zhuang and coworkers mixed curcumin with EVs derived from a T-cell lymphoma cell line and showed that these EVs could be taken up by microglial cells in the brain after intranasal administration and could ameliorate LPS-induced brain inflammation, in contrast to free curcumin [56]. Intriguingly, these effects were only observed for small EVs (<100 nm) but not for bigger EVs (500–1000 nm), suggesting that the brain-translocating capacity of EVs is restricted to specific EV subpopulations. Using a similar approach, the authors showed that a hydrophobic Stat3-inhibitor mixed with small EVs could inhibit growth of xenografted brain tumors. The advantages of encapsulation of curcumin and other poorly soluble molecules in EVs have also been highlighted by others. For example, it was shown that curcumin could readily

be incorporated in raw bovine milk-derived EVs by simple mixing, improving its systemic bioavailability after oral administration to rats compared with free curcumin [57]. Similarly, brain delivery and anti-inflammatory activity of curcumin could be improved by its incorporation in surface-modified bone marrow mesenchymal stem cell (BM-MSC)-derived EVs [53]. A number of recent studies revealed that the effects of curcumin could also be potentiated by pretreating the EV-producing cells with curcumin prior to EV isolation. Such curcumin-laden EVs were shown to deliver curcumin to the brains of rats after ischemia–reperfusion injury [58] or after induction of an Alzheimer's disease phenotype [59] and ameliorated disease phenotypes. Additionally, EVs from curcumin-treated BM-MSCs reduced chondrocyte apoptosis in a mouse model for osteoarthritis [60]. Of note, the effects in the latter studies could be explained by an altered composition of the EVs itself rather than the incorporation of curcumin in EVs, as cells adopt an anti-inflammatory phenotype upon curcumin treatment, which is likely reflected in the composition – and biological effects – of the resulting EVs [61]. Nevertheless, most of the aforementioned studies convincingly showed that the stability and bioavailability of curcumin is greatly improved by its incorporation in EVs. Of note, it is traditionally challenging to stably incorporate hydrophobic drug molecules in liposomes due to their rapid extraction by hydrophobic compartments in biological fluids (e.g. lipoproteins and/or EVs in plasma) [43]. It remains to be investigated whether such drug exchange between hydrophobic carriers also occurs in EV-based formulations of hydrophobic drugs.

3.2. Doxorubicin as a model hydrophilic drug molecule

Similar effects have also been shown for water-soluble drug molecules. An often used example is doxorubicin, a chemotherapeutic agent which is clinically used in liposomal formulations (Caelyx/Doxil and Myocet) to improve its side effect profile. Several studies have shown that this effect can also be achieved by incorporation of doxorubicin in EVs. Doxorubicin has been loaded in EVs derived from a variety of sources using electroporation and the resulting formulation exhibited reduced cardiac toxicity after intraperitoneal, intratumoral or intravenous administration compared to free doxorubicin [62–65]. Interestingly, a recent study showed that doxorubicin-loaded HEK293-derived EVs were taken up more efficiently by a range of recipient cells than clinically used liposomal doxorubicin formulations (including the non-PEGylated Myocet), resulting in a lower IC50 [66]. This suggested that EVs could improve the therapeutic window of doxorubicin. Unfortunately, evidence for such potential benefit of EVs over clinically approved doxorubicin formulations (which are optimized to avoid uptake by phagocytic cells) is lacking as most studies compare efficacy of doxorubicin-loaded EVs with free doxorubicin. A remarkable study showed some ground for this phenomenon by comparing the biodistribution and antitumor effects of Doxil after it had been extruded together with EVs [37]. Strikingly, after repeated intravenous administration the resulting "hybrid" particles accumulated better in tumor tissue than Doxil. Additionally, the hybrid particles inhibited tumor growth to a similar or greater extent than Doxil, depending on the type of EVs used in the extrusion process. In a similar example, doxorubicin-loaded porous silicon nanoparticles were incubated with cancer cells, which subsequently secreted the particles inside EV-like membranes [67]. The EV-encapsulated doxorubicin nanoparticles accumulated to a higher extent in tumor tissue after intravenous administration, inhibited tumor growth in three different tumor models, and was more effective at inhibiting metastasis than "naked" doxorubicin nanoparticles or 8-fold higher doses of free doxorubicin. The possibilities to load a variety of small molecule cargoes in EVs via hybridization with existing drug delivery systems was also explored by Piffoux and coworkers [68]. They elegantly showed that fusion between drug-loaded liposomes and EVs could be induced using PEG as a molecular crowding agent, resulting in hybrid particles which showed enhanced *in vitro* uptake and photosensitizer delivery to cells compared with photosensitizer-loaded liposomes.

These studies suggest that the presence of EV-components on existing drug delivery systems can improve their efficacy. In a provocative study it was shown that this effect may not be restricted to EV-components but may be extended to cellular components in general. The authors prepared EV-like nanovesicles by extruding cells through a series of membranes with pore sizes of 10, 5 and 1 μm in the presence of doxorubicin. The resulting particles accumulated in syngeneic tumors in mice after repeated intravenous administration and showed slightly better tumor growth inhibition than liposomal doxorubicin [69].

3.3. Other small molecules

While the largest body of evidence for the potential benefit of EVs for the delivery of small molecules governs doxorubicin and curcumin cargoes, loading of other small molecules has also been explored. Depending on a small molecule's physicochemical properties, loading in EVs can be accomplished relatively straightforward via simple mixing and a short incubation. This is mostly true for lipophilic molecules which presumably associate with the EV membrane (e.g. paxlitaxel [70–72], curcumin), but this loading process may also be amenable to some water-soluble molecules (e.g. antocyanidins [73]), which may associate with EV surface proteins or glycans. In slight contrast with this observation, a comparative study revealed that the hydrophobicity of small molecules can be used to predict the efficiency of spontaneous association with EVs. Hydrophobic porphyrins were loaded in EVs from different cell sources upon simple incubation, whereas hydrophilic phorphyrins required electroporation or permeabilisation of the EV membranes by other means (i.e. extrusion, hypotonic dialysis or saponin treatment) [74]. Interestingly, the lipid composition of the EVs (which differed between EV sources) was found to also impact the degree of small molecule loading, suggesting that the EV producing cell needs to be carefully selected for each to-be-loaded small molecule [74].

Others have also shown that efficient loading of water-soluble molecules may require physical disruption of the EV membrane. For example, sonication was shown to be required to load the antibiotic vancomycin in EVs [75], whereas electroporation was used to encapsulate Olaparib [76]. It is conceivable that the temporary disruption of the EV membrane induced by such methods results in some loss of EV content or altered size distributions (for example due to aggregation as has been observed in specific electroporation setups [77,78]), which could have pronounced effects on EV biodistribution, cellular uptake and intracellular processing. As an alternative, some molecules such as oxaliplatin can be chemically linked to the surface proteins of EVs [79], or, as shown for piceatannol, actively transported into vesicles derived from disrupted cells by the use of pH gradients [80]. However, the latter approach is not readily applicable in naturally secreted EVs in which the internal pH or salt concentrations cannot be easily modified without compromising EV integrity and content [81].

In order to circumvent the potential non-reversible damage that such methods may elicit in the EVs, cell-based loading strategies can be employed as well. For example, Tang and coworkers showed that treatment of tumor cells with chemotherapeutics, such as methotrexate, doxorubicin, cisplatin and paclitaxel, induced apoptosis and resulted in the release of large EVs (presumably apoptotic bodies) encapsulating the selected compounds. The drug-loaded

Table 1
Overview of studies discussed in this review in which small molecules were loaded in EVs to increase their efficacy and/or reduce their toxicity.

Compound	EV source	Loading method	Administration route and species	Observed effects compared to free drug	Reference
Curcumin	EL-4 T-cell lymphoma cells	Mixing and incubation at 22 °C for 5 min	Intrapertioneally in mice	↓ Serum levels of IL-6 and TNF-α and mortality after LPS-induced septic shock	[55]
	EL-4 T-cell lymphoma cells	Mixing and incubation at 22 °C for 5 min	Intranasally in mice	↓ IL-1β secretion by microglia ↑ Microglia apoptosis after LPS challenge ↓ Clinical score of experimental autoimmune encephalomyelitis	[56]
	Raw bovine milk	Mixing and incubation at 22 °C	Oral gavage in rats and mice	↑ Distribution to liver, lungs and brain of rats ↓ Xenografted breast cancer tumor growth in athymic nude mice	[57]
	Mouse bone marrow-derived MSCs	Surface-modification of EVs with RGDyK peptides, loaded by mixing and incubation at 22 °C for 5 min	Intravenously in mice	↑ Uptake by ischemic brain regions after ischemia–reperfusion injury ↓ TNF-α, IL-1β, IL-6, p-p65 and cleaved Caspase-3 expression in ischemic region	[53]
	RAW264.7 macrophages	Cellular incubation with curcumin for 24 h	Intravenously in rats	↑ Accumulation in brain ischemic regions after ischemia–reperfusion injury, ↓ Brain oxidative stress, ischemic area and neurological function ↓ Blood-brain barrier permeability	[58]
	RAW264.7 macrophages	Cellular incubation with curcumin for 24 h	Intraperitoneally in mice	↑ Accumulation in brains after okadaic acid-induced Tau hyperphosphorylation ↑ Cognitive function ↓ Tau hyperphosphorylation	[83]
	MSCs (origin not reported)	Cellular incubation with curcumin	Unknown administration route in mice	↓ Chondrocyte apoptosis compared with EVs without curcumin	[60]
Doxorubicin	MDA-MB-231 cells	Electroporation	Intraperitoneally in mice	↓ Cardiac toxicity ↑ Tolerability for doxorubicin ↓ Xenografted MDA-MB-231 tumor growth	[62]
	HEK293 cells	Electroporation	Intratumorally in mice	↓ Xenografted 4 T1 tumor growth ↓ Cardiac toxicity	[63]
	Immature mouse dendritic cells	Electroporation	Intravenously in mice	↓ Xenografted MDA-MB-231 tumor growth ↓ Cardiac toxicity	[64]
	HEK293 cells	Electroporation	Only tested *in vitro*	↑ Uptake in various cell lines ↓ IC50 values compared to liposomal doxorubicin	[66]
	Human and murine red blood cells	Electroporation	Intravenously in mice	↓ Growth of orthotopic liver tumors ↓ Cardiac toxicity compared to ~ 15 fold higher doses of free doxorubicin	[65]
	HT1080 tumors	Extrusion with Doxil	Intravenously in mice	↑ Accumulation in xenografted HT1080 tumors ↓ Tumor growth compared to Doxil	[37]
	H22, Bel7402, or B16-F10 cells	Incubation of cells with doxorubicin-loaded porous silicon nanoparticles for 6 h	Intravenously in mice	↑ Tumor accumulation and penetration ↓ Tumor growth compared to "naked" doxorubicin-loaded porous silicon nanoparticles	[67]
	RAW264.7 macrophages	Extrusion of cells with doxorubicin	Intravenously in mice	↓ Tumor growth in a syngeneic mouse model compared to liposomal doxorubicin	[69]
Paclitaxel	PC-3 cells	Mixing and incubation for 1 h at 22 °C	Only tested *in vitro*	↑ Cytotoxic activity on autologous cancer cells	[70]
	Embryonic stem cells	Mixing and incubation with surface-modified EVs for 2 h at 22 °C	Intravenously in mice	↓ Xenografted subcutaneous U87 tumor growth ↑ Survival of orthotopic brain tumor mouse model	[71]
	LL/2 cells	Mixing and incubation for 1 h at 22 °C	Intravenously in mice	↑ Localized paclitaxel-induced inflammation in tumors in immunocompetent mice	[72]
Photosensitizer (mTHPC)	Mouse MSC cell line (C3H)	PEG-induced fusion with mTHPC-loaded liposomes	Only tested *in vitro*	↑ Uptake by cancer cell lines and tumor spheroids ↓ Uptake by macrophages after EV fusion with PEGylated liposomes	[68]
Anthocyanidins	Raw bovine milk	Mixing and incubation for 15 min at 22 °C	Oral gavage in mice	↓ Growth of xenografted tumors in immunodeficient mice	[73]
Vancomycin	RAW264.7 macrophages	Sonication	Intravenously in mice	↑ Intracellular antibacterial effects	[75]
Porphyrins	MDA-MB-231, HUVEC, human BM-MSCs, human embryonic stem cells	Electroporation, mixing and incubation, saponin treatment, hypotonic dialysis	Only tested *in vitro*	↑ Cellular uptake and phototoxicity compared to free drug or liposomal porphyrins	[74]
Olaparib	Hypoxic MDA-MB-231 cells	Electroporation	Intratumorally in mice	Similar efficacy as free drug in inhibiting xenografted MDA-MB-231 tumor growth	[76]
Oxaliplatin	BM-MSCs	Chemical conjugation	Intravenously in mice	↓ Orthotopic pancreatic tumor growth in immunocompetent mice	[79]

Table 1 (*continued*)

Compound	EV source	Loading method	Administration route and species	Observed effects compared to free drug	Reference
Piceatannol	Differentiated HL60 cells	pH gradient established in vesicles derived from disrupted cells	Intravenously in mice	↓ Acute lung inflammation after LPS challenge ↑ Survival after LPS-induced septic shock	[80]
Methotrexate, doxorubicin, paclitaxel, cisplatin	H22 and A2780 cells	Incubation of cells with chemotherapeutics	Intraperitoneally or intravenously in mice	↑ Survival of immunocompetent mice with intraperitoneally xenografed H22 tumors after methotrexate-EV treatment ↓ Ovarian tumor growth in immunodeficient mice after cisplatin-EV treatment ↓ Subcutaneously xenografted H22 tumor growth after doxorubicin-EV treatment	[82]

particles were found to be equally effective for the treatment of established tumors as liposomal counterparts, but showed reduced toxicity [82]. The risk of such approaches is that, as a cellular defense mechanism, the composition of the EVs may change towards a tumor growth-promoting phenotype. For instance, it was recently demonstrated that doxorubicin treatment can result in increased CD44 expression in EVs, which can promote resistance to chemotherapy in cells taking up the EVs [83]. Similar mechanisms have been described for other chemotherapeutics [84], showing that the use of tumor-derived EVs for delivery of therapeutics should be approached with caution.

3.4. Challenges associated with EV-based drug carriers

Taken together, a substantial number of studies have explored the possibilities to load EVs with small molecules in order to improve their bioavailability, safety and/or biodistribution towards specific tissues or cell types (examples mentioned in this review have been summarized in Table 1). It is clear that EVs can improve these parameters. However, clinical translation of such EV-based drug formulations is still extremely challenging. One of the major hurdles is the large-scale and reproducible production of EVs, as lab-scale cell cultures typically do not produce quantities of EVs sufficient for further manufacturing processes (especially compared to the ease of production of liposomes or other synthetic drug delivery systems) and EV composition can vary based on cell state and cell type. In addition, efficient loading of EVs with drug molecules without affecting their functional properties remains a formidable challenge [85]. For molecules that spontaneously associate with EVs, such as curcumin, it is to be expected that EV integrity is largely or completely maintained. However, as discussed previously, this may come at the cost of rapid drug extraction in bodily fluids. For (hydrophilic) compounds loaded in the EV lumen, such rapid extraction is prevented by the EV membrane. However, it remains challenging to achieve sufficient drug loading in these cases. Drug loading may be enhanced by EV membrane perturbation, e.g. using electroporation or sonication, but such methods may result in non-reversible EV damage or aggregation, affecting their drug delivery capacity. Cell-based loading methods could be used to circumvent this, but these generally result in lower drug encapsulation efficiency as drug loading would mostly be diffusion-driven process, which is limited by the dose tolerated by the cells. Furthermore, it is likely that EV composition changes when EV-producing cells are incubated with pharmacologically active compounds. Such altered composition may significantly affect EV behavior and their capacity to deliver compounds to the intended cells or organs. Furthermore, the major benefit of using EVs over liposomal counterparts appears to reside in their presumed biocompatibility, ability to cross biological barriers and potential specificity for cells or organs. However, the evidence for these benefits is still scarce, as head-to-head comparisons of EVs with liposomes in the context of these critical pharmaceutical

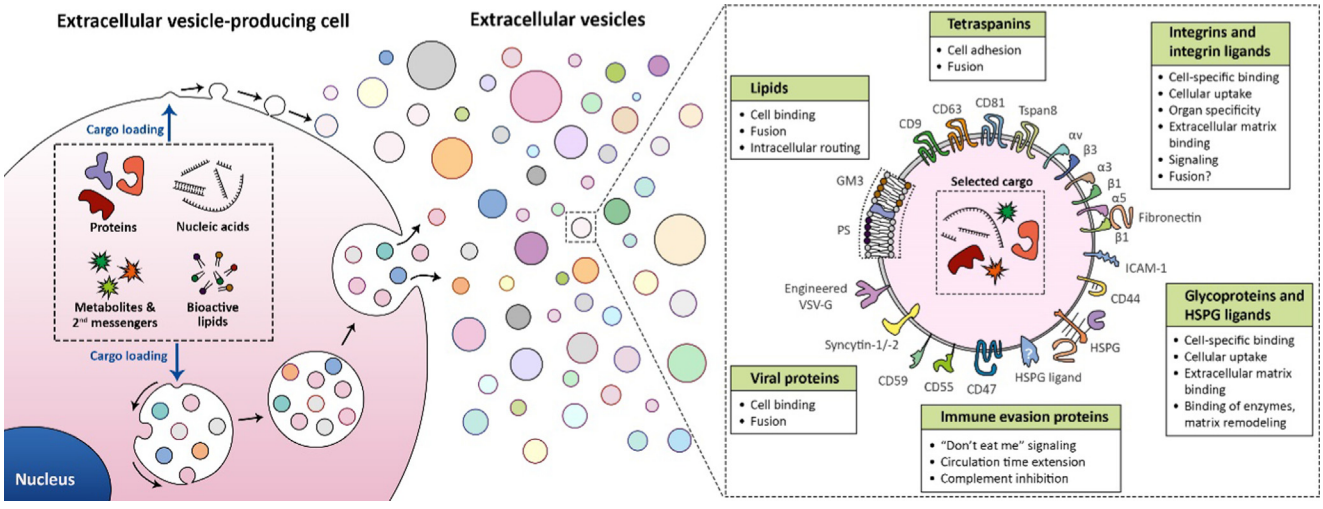

Fig. 1. Overview of EV surface molecules discussed in this review, which could be directly or indirectly involved in functional EV cargo delivery. Cells secrete EVs derived from the plasma membrane or multivesicular bodies, which are actively or passively loaded with cellular components. Secreted EVs are highly heterogeneous in terms of size and content, and selected subpopulations may be enriched in specific surface components that facilitate cargo delivery (shown in inset on the right). Abbreviations: HSPG: heparan sulfate proteoglycans; PS: phosphatidylserine; GM3: ganglioside GM3; VSV-G: vesicular stomatitis virus G protein.

attributes are rarely made. Therefore, it remains to be investigated whether the benefits of EVs for the delivery of small molecules outweigh the challenges associated with their production.

4. The EV "surfaceome as mediator of delivery of biomolecules

Whereas small molecules often do not require intracellular delivery by a suitable delivery system to exert their function, intracellular delivery is mandatory for the functionality of therapeutic nucleic acids (e.g. siRNA, mRNA) and most therapeutic proteins (e.g. Cas9-guide RNA complexes for gene editing). Despite ongoing advances in the design of drug delivery systems, efficient and safe intracellular delivery of these molecules remains a formidable challenge [86]. As EVs are naturally packaged with these biomolecules and can functionally transfer these to recipient cells, their use for the delivery of biomolecules is intensely investigated (for a number of excellent reviews on the topic we refer to [21,87,88]). Despite the rapidly growing interest to exploit EVs for the delivery of biologicals, surprisingly little is known about the underlying mechanisms by which EVs can functionally transfer their cargo to recipient cells. It is conceivable that this activity is orchestrated by a complex interplay of surface molecules on EVs (also known as the EV "surfaceome") and their environment, including the extracellular matrix and (plasma- and endosomal) membranes of recipient cells. Furthermore, it is known that the surface signature of EVs is highly heterogeneous (even within a pool of isolated EVs from a single cell source [24,89]), which could reflect different functionalities. For instance, after internalization of EVs, some subpopulations could be trafficked to lysosomes for degradation while others may fuse with endosomal membranes and release their content. Understanding which effectors of the EV "surfaceome" drive such functional content transfer is of critical importance to fully exploit their use as drug delivery vehicles and can aid in the design of novel EV-inspired drug delivery systems [25]. In the following sections, we will discuss and speculate on EV surface molecules potentially involved in content transfer in recipient cells, which are summarized in Fig. 1. We do not intend to be exhaustive in this regard as the biogenesis and surface composition of EVs has been extensively reviewed by others (we refer to [2,90,91] for excellent reviews on the topic), but aim to highlight interesting findings and provide leads for further research.

4.1. Tetraspanins: More than EV assembly machinery?

The biogenesis of EVs is a complex process which is not yet fully understood. EV secretion from multivesicular bodies was initially believed to be dependent on the endosomal sorting complex required for transport (ESCRT), a family of four protein complexes which orchestrate a cascade of events in which specific ubiquitinated proteins are recruited to microdomains on endosomes, which can bud inwards and form intraluminal vesicles [2]. The resulting multivesicular bodies (MVBs) can fuse with lysosomes for degradation, or fuse with the plasma membrane to release their intraluminal vesicles into the extracellular milieu (after which they can be termed exosomes). However, knockdown studies have established that MVBs can be formed in the absence of all four ESCRT proteins [92] and that EV formation can also be modulated by other proteins, such as sphingomyelinases and their metabolites [93] or by the tetraspanin CD63 [94]. Other tetraspanins, such as CD9, CD81 and CD82 are also involved EV biogenesis, specifically at the level of cargo recruitment to EVs [95,96]. Due to their participation in EV biogenesis, many of the abovementioned proteins are often found to be enriched in EV preparations. Examples are the ESCRT protein Tsg101, its interaction partner Alix, and the tetraspanins CD9, CD63 and CD81. Because of their high abundance in EVs, the EV research community has coined these proteins as canonical EV markers, which can be used to demonstrate the presence of EVs in a variety of assays [97]. Interestingly, increasing evidence is emerging that tetraspanins do not only function at the level of EV biogenesis, but may also play direct or indirect roles in EV-mediated cargo transfer in recipient cells.

One of the tetraspanins that is well studied is CD9. In an early study, Le Naour and coworkers knocked out CD9 in mice to study its role in cancer metastasis (as CD9 is highly expressed in cancer cells), and discovered that female mice of the generated strain were almost infertile [98]. These observations were shared by others [99], and it was established that, while sperm cells could still bind to the surface of oocytes which lacked CD9, they could no longer fuse and promote fertilization. This could be restored by expression of CD9 (both human and murine) on the surface of the oocytes, as well as by the expression of mouse CD81 [100]. Interestingly, the addition of oocyte-derived CD9-expressing EVs to a mixture of CD9 knockout sperm cells and oocytes has also been shown to promote sperm fusion with the oocytes [101]. The authors speculated that the EVs could be taken up by, or fuse with the sperm cells, thereby transferring CD9 and improving the ability of the sperm to fuse with oocytes. Furthermore, CD9 and CD81 have been implicated in fusogenic processes in murine C2C12 myoblasts, as antibodies against CD9 and CD81 inhibited cell–cell fusion [102]. Although these early studies focused on the role of CD9 and CD81 on cell membranes, such observations could also hint towards roles of tetraspanins in the fusion of EVs with either endosomal membranes or plasma membranes of recipient cells. Interestingly, it was reported that knockdown of CD9 in EVs from two different cancer cells abolished their uptake by recipient cells. Likewise, knockdown of CD9 in the recipient cells, but not EVs, resulted in the same effect [103]. Though these results suggest that the presence of CD9 on EVs is critical for EV uptake, it should be noted that CD9 knockdown likely influences the expression levels of other proteins in cells and EVs, which could have indirectly contributed to the observed effects. The same group recently also showed that addition of Fab fragments of CD9 antibodies to an EV-cell coculture inhibited EV uptake, whereas the full-length CD9 antibodies surprisingly *promoted* EV uptake [104]. The authors speculated that EV uptake may therefore be dependent on the interaction of CD9 on the surface of both EVs and recipient cells. Other studies have also demonstrated that CD9 on EV surfaces could facilitate EV uptake. For example, Böker and colleagues showed that the infectivity of EV-encapsulated lentiviral particles could be increased by overexpression of CD9 in the virus-producing cells [105]. Furthermore tetraspanins CD9, CD63 and CD81 and their associated membrane microdomains have been implicated as "gateways" for infection of numerous other viruses and bacteria as well [106,107], though it should be noted that such functionalities have mainly been appointed to tetraspanins on the surface of cells rather than on the surface of infectious particles. In contrast, an interesting recent study of Joshi *et al* closely examined the uptake of EVs and the fate of their cargo in recipient cells, and elegantly showed that the protein content of CD63-bearing EVs can be released from endosomes after EV uptake. Though this does not prove that CD63 is responsible for EV fusion at the endosomal membrane, it indicates that CD63 may be an attractive anchor for the cytosolic delivery of proteins using EVs, provided that a suitable release mechanism is employed as CD63 itself remains endosome-associated [108]. However, this principle is still subject to debate, as a recent report showed that β-lactamase fused to CD63 could hardly be delivered to the cytoplasm of recipient cells in the short timeframe in which EVs are taken up (within 4 h), in a large variety of EV donor-recipient cell combinations [109].

Together, these studies support roles for tetraspanins in the association of EVs with recipient cells, and potentially in release

of their cargo. However, evidence for the latter is yet circumstantial and mostly based on cells rather than EVs. In addition, it could be that the discussed roles of tetraspanins in membrane fusion are heavily cell-type dependent. Furthermore, as tetraspanins are known to sequester other cell-adhesion proteins such as integrins in EVs [110,111], it is challenging to study the role of individual roles of tetraspanins in EV-mediated cargo transfer. Further tetraspanin overexpression/knockdown studies focused on the fate of EVs in recipient cells could provide additional insights in this matter. However, such studies should be approached with caution as the manipulation of tetraspanin levels in EV-secreting cells could interfere with EV biogenesis, resulting in impaired EV secretion or altered EV composition. The use of appropriate controls and in-depth EV characterization techniques (e.g. proteomic and/or lipidomic analyses) are strongly recommended to avoid such confounding factors and elucidate the functional role of tetraspanins in EV-cell interactions.

4.2. Cell-binding proteins

4.2.1. Integrins

One hallmark of tetraspanins is that they form complexes with each other and with other proteins in tetraspanin-enriched microdomains (which are distinct from lipid rafts) on cellular membranes [112]. One dominant type of tetraspanin interaction partners are integrins [113]. These molecules are – presumably due to their association with tetraspanins [114] - frequently identified in EV preparations, and endow EVs with adhesive properties that allow binding to specific cells and extracellular matrix components. This interaction may precede EV internalization and potential cargo delivery by recipient cells. For example, Al-Dossary and coworkers elegantly showed that EVs derived from murine oviductal fluid express the αv subunit of αvβ3 integrin, which could bind to the same integrin on mouse sperm [115]. The resulting interaction induced fusion of the EVs with the sperm, which could be inhibited by typical integrin ligands, including RGD peptides, vitronectin and anti-αv antibodies. The same effect was observed for fibronectin, a ligand for α5β1 integrin, suggesting involvement of multiple integrins in this process. Integrins on EVs have also been implicated in the onset of cancer metastasis. Seminal work of Lyden and coworkers revealed that EV biodistribution after systemic administration differed between EVs from various cancer cell lines. Furthermore, uptake of EVs in those organs could create a "pre-metastatic nice" which favored growth of metastases upon intravenous injection of the respective tumor cell lines [38]. Additionally, the authors showed that EV uptake (and associated metastasis) in the lungs and liver could be reduced by knocking down or blocking integrin subunits β4 and β5, respectively, on the EVs. These results suggest that EVs use integrins to localize to specific tissues by binding integrin ligands (such as extracellular matrix proteins laminin or fibronectin) or other integrins on specific cells.

The importance of integrins for the functionality of EVs in disease progression has also been shown by others. For example, it was recently shown that the uptake of EVs derived from mesenchymal stem cells from multiple myeloma patients by cultured multiple myeloma cells was dependent on integrins α3 and β1 on the surface of EVs, and that EV expression of α3 correlated with disease severity in multiple myeloma patients [116]. EV surface integrins play similar cell-binding roles in immunology. For example, T-cells stimulated by retinoic acid (a dendritic cell metabolite) release EVs expressing α4β7, which promotes EV homing to the small intestine where its ligand MadCAM-1 is expressed [117], whereas integrin β2 promotes adhesion of activated mononuclear cell-derived EVs to lung epithelial cells, promoting inflammation [118]. On the other hand, integrin β3 may be involved in binding and uptake of EVs by $Ly6C^{high}$ monocytes in the lung [119]. Integrin β1 (also known as CD29), but not integrins α4 and α5 were shown to promote binding and internalization of mesenchymal stem cell-derived EVs to kidney tubular epithelial cells [120], whereas integrin α4 in concert with β1 was required for internalization of human liver stem cell-derived EVs by liver cancer cells [121].

Together, these examples highlight that EVs rely on expression of integrins for their interaction with recipient cells and organs. However, these effects may be restricted to specific integrin subclasses or EV-cell combinations, as inhibition of integrin β3 and αV on EVs failed to impact EV-cell association *in vitro* [122,123].

4.2.2. Integrin ligands

Integrin-mediated EV-cell interactions can also occur reciprocally, as exemplified by the expression of integrin ligand laminin γ2 on the surface of EVs derived from metastatic oral squamous carcinoma cells. Uptake of these EVs by lymphatic endothelial cells has been shown to be dependent on the cellular expression of integrin α3, and knockdown of EV-expressed laminin y2 decreased EV drainage to lymph nodes after intratumoral administration [124]. In a similar fashion, EVs derived from dendritic cells can bind LFA-1 on activated T-cells, presumably via EV-expression of its ligand ICAM-1 [125]. In addition, both ICAM-1 and LFA-1 are secreted on EVs of activated cytotoxic T-cells, and thereby bind dendritic cells leading to immunosuppression [126]. This integrin LFA-1-based EV-cell interaction has also been documented in other cell types. For example, macrophage-derived EVs have been shown to express LFA-1 and bind to ICAM-1 on brain microvascular endothelial cells [59]. Furthermore, EVs of several types of cancer have been shown to express ICAM-1 and integrins such as αvβ6 and αvβ3, which may play roles in their ability to cross the blood–brain barrier [127]. Recently it was shown that also EVs from human umbilical vein cells (HUVECs) express ICAM-1, and EV subpopulations carrying this ligand promote migration of THP-1 monocytes and drive ICAM-1 expression on recipient HUVECs [128]. Of interest, our group recently showed that integrin β1 on HEK293T cells was involved in the functional uptake of sgRNA carried by MDA-MB-231 EVs, suggesting that integrin ligands are present on EV subclasses which can functionally transfer RNA [129].

Besides facilitating EV interactions with recipient cells, integrins also play important roles in EV interactions with the extracellular matrix [130]. Sung and coworkers showed that EVs from fibrosarcoma cells contain both integrin α5β1 and its ligand fibronectin, and postulate that via this interaction EVs form a scaffold with the extracellular matrix over which cancer cells can migrate [131]. Similarly, EVs containing integrin αV were found to stimulate the adhesion of prostate cancer cells to vitronectin and increase their tumorigenesis *in vivo* [132]. Likewise, platelet-derived EVs were found to bind integrins on endothelial cells, and provide a scaffold for neutrophil binding to these cells [133]. Following the same mechanism, platelets can adhere to endothelial cells via a scaffold of unidentified RGD peptide-expressing EVs released in the circulation after ischemic stroke [134].

4.2.3. Glycoproteins

Other adhesion molecules may also be involved in EV-cell interactions. For example, EVs derived from mesenchymal stem cells and various types of cancer cells have been shown to express the hyaluronic acid binding glycoprotein CD44, which is expressed mainly at the cell's plasma membrane [120,135–139]. Binding and uptake of these EVs (presumably microvesicles) to their target cells can be inhibited by soluble hyaluronic acid [120,135], by knockdown of CD44 [139] or by digestion with hyaluronidase [140]. The interaction of CD44 with hyaluronic acid also allows EVs to interact with the extracellular matrix [141] with potential implications for cancer cell metastasis [130,142,143].

Besides CD44, also other glycoproteins have been reported to be involved in EV-cell interactions. For example, heparan sulfate-coupled proteoglycans (HSPGs) at the blood brain barrier endothelium were recently shown to be involved in EV uptake, as heparin (a heavily sulfated polysaccharide) or Heparinase III (an enzyme that degrades heparan sulfate) abolished the uptake of neural stem cell-derived EVs [144]. Using similar methodology, EVs have also been shown to bind HSPGs on a variety of other cell types [122,145–151]. Interestingly, HSPGs have also been found on the surface of EVs, but were shown to not be involved in EV binding to recipient cells [147]. Instead, EV surface HSPGs could sequester growth factors such as vascular endothelial growth factor (VEGF) and facilitate their transfer to recipient cells [152]. In a similar fashion, EV surface HSPGs may bind other molecules such as secreted metalloproteases [153] and other enzymes [154] and potentiate their functionality at specific extracellular locations.

It is yet unclear which molecules on EVs are responsible for their interaction with HSPGs on recipient cells. A recent study provided some insights into this matter and showed that EVs from the parasite *Trichomonas vaginalis* express the enzyme 4-α-glucanotransferase. This carbohydrate-binding protein was found to bind HSPGs on human prostate (BPH-1) cells and promote EV uptake [155]. In eukaryotic systems, EVs have been shown to contain surface-bound histones [156,157], which have been suggested to bind to cellular HSPGs to promote EV internalization [158], though direct evidence of this interaction is still lacking.

Additionally, multiple studies have shown that HSPGs on the EV surface can bind fibronectin, which could act as a scaffold for EV binding to HSPGs on the surface of recipient cells [159–161]. Interestingly, EV-expressed ligands for cell surface HSPGs may not be present on EVs from any source. Di Noto and coworkers used surface plasmon resonance to show that EVs derived from plasma of multiple myeloma patients adhered better to immobilized heparin than EVs from monoclonal gammopathy of undetermined significance patients or healthy individuals[162]. It is tempting to speculate that the surface expression pattern of HSPG ligands on specific EV subtypes is linked to their functionality, which may include cargo transfer. However, to date the interaction of EVs with cell surface HSPGs has mostly been studied at the level of EV-cell interaction and subsequent internalization *in vitro*. It remains to be investigated to what extent these interactions also occur *in vivo* and whether EV-bound HSPG ligands can promote (or contribute to) EV cargo transfer in recipient cells. As HSPGs have been shown to associate with the ESCRT protein Alix to sort cargo to EVs during EV biogenesis [163,164], a role in cargo release in recipient cells can certainly be envisioned.

Taken together, there is a vast body of evidence that integrins, other adhesion molecules and their ligands are expressed on the surface of EVs from a large variety of cell types. The interaction of these molecules with their binding partners allows EVs to adhere to cells and organs *in vivo* and exert their functions, which can involve integrin-based downstream signaling (e.g. as shown by Chanda et al. [165]. However, it appears unlikely that such adhesion molecules also play direct roles in cargo transfer of EVs. EV binding to cells is often followed by EV internalization, after which other molecules in the EV surfaceome promote cargo release or – in the absence of such molecules – EVs are destined for lysosomal degradation. Further research is needed to establish whether adhesion molecules on EVs have functionalities in this process or whether their function is limited to the first steps of EV-cell engagement.

4.3. Immune evasion proteins

As mentioned before, one of the reasons that EVs have attracted interest for drug delivery applications, is their supposed intrinsic capacity to avoid recognition by the immune system, which may especially be true for autologous EVs. Such "stealth" behavior is often desired for drug delivery systems, and is generally achieved for synthetic nanoparticles through surface functionalization with polyethylene glycol (PEG). This reduces nanoparticle opsonization and rapid clearance from circulation, but has drawbacks such as reduced cellular uptake and potential occurrence of antibody-mediated hypersensitivity reactions [166]. Although we and others have shown that exogenous EVs are also rapidly cleared from circulation by macrophages after systemic administration [45,167–170], adverse reactions to EVs have not been documented in most studies. Of note, extremely high doses of tumor-derived EVs have been reported to cause asphyxiation after intravenous administration to mice, which likely resulted from EV aggregation and subsequent obstruction of microvasculature in the lungs [46]. The observed problems may have been induced by the ultracentrifugation protocol used to isolate EVs (as reported by others to promote EV aggregation and lung accumulation [171]) rather than the EVs' intrinsic properties. In contrast, a number of molecules have been described to prevent EV recognition and clearance by the immune system.

An interesting study of Kamerkar and coworkers showed that EVs from fibroblasts express the integrin-associated protein CD47 [172]. This protein interacts with SIRPα (CD172a) on myeloid cells and conveys a 'don't eat me' signal, preventing phagocytosis [173]. By this mechanism, EVs expressing CD47 were shown to exhibit prolonged circulation times and decreased uptake by circulating monocytes after intraperitoneal injection compared to 100 nm liposomes (presumably composed of 70:30 PC:Cholesterol, although the exact composition was not reported). Furthermore, this effect was abolished when the interaction between CD47 and SIRPα was blocked using antibodies, and increased upon overexpression of CD47 on the EVs. The CD47 expressing EVs could subsequently be exploited to deliver siRNA to pancreatic tumors with remarkable efficiency (especially when compared to clinical-grade siRNA formulations [174]) [172]. The "don't eat me" signaling properties of CD47 on EV membranes were recently also shown to slightly prolong circulation time and tumor accumulation of liposome-EV hybrids [175]. In a reciprocal fashion, extrusion-based nanovesicles and EVs obtained from SIRPα-overexpressing cells have been shown to block CD47 on tumor cells and promote immune recognition and tumor eradication [176–178].

The group of McLellan has also demonstrated that EVs could have immune evasive properties through surface display of sialic acids. In two interesting studies, it was shown that B-cell derived EVs display α2,3-linked sialic acids which interact with CD169 (Siglec-1) on macrophages in spleen and lymph node, suppressing T-cell responses to EV-presented tumor antigens [179,180]. Sialic acid residues on glioblastoma-derived EVs have been suggested to convey similar immunosuppressive signals to dendritic cells [181]. In addition, T-cell activation can be suppressed by sialic acid-bearing EVs from ovarian tumor ascites [182]. However, the contribution of these sialic acids on EVs to their immune-evasive capacity appeared to be relatively small compared to the effects documented for CD47; treatment of EVs with sialidase (removing sialic acid residues on the EV surface) has been shown to lower EV macrophage binding in the spleen, but this procedure did not markedly affect EV circulation time or whole-body biodistribution [180,183].

EVs also express other molecules that may assist in immune evasion. For example, expression of PD-L1 on glioblastoma-derived EVs has been shown to suppress T-cell activation by engagement of PD-1 on the T-cell surface [184]. This EV-based PD-1/PD-L1 signaling process appears to be a general immune-evasion mechanism shared by other types of cancer as well

[185], and has been coined as a therapeutic target [186,187]. However, there are so far no reports showing that the function of PD-1 on EVs goes beyond such signaling events (e.g. extending the circulation time of EVs). Hence, the expression of PD-1 on EV membranes appears to have limited value for drug delivery purposes.

A set of molecules worth mentioning in this context are CD55 and CD59. An early study of Clayton and coworkers showed that the expression of these glycosylphosphatidylinositol (GPI)-linked proteins on the membranes of antigen presenting cell-derived EVs rendered the EVs immune to complement-mediated lysis [188]. Likewise, shedding of CD55 and CD59 through EVs has been postulated to increase vulnerability of EV-secreting cells to complement-mediated lysis in the context of inflammation or red blood cell ageing [189,190]. Unfortunately, follow-up studies examining the roles of these complement-regulating proteins on the surface of EVs are lacking. Hence, it is unclear to what extent complement-mediated EV lysis also occurs *in vivo* and whether this can be prevented by surface expression of CD55 and CD59.

4.4. EV membrane lipids

Lipids are an important structural component of EVs, but can also assist in EV-mediated cargo transfer. Several studies have shown that EV membranes are enriched in specific lipids compared to their parent cells and that the EV membrane lipid composition is dynamic, reflecting the state of the parent cell or host organism [191–196]. For example, a recent study uncovered that EVs from plasma of COVID-19 patients contain higher levels of ganglioside M3 (GM3) and phosphatidylserine (PS) compared to EVs from plasma of healthy controls [197]. Moreover, the lipid composition of EVs differs between smaller EVs (likely originating from MVBs) and their plasma membrane-derived counterparts; small EVs have been reported to be enriched in cholesterol, glycolipids and free fatty acids, whereas larger EVs have shown enrichment for PS, ceramides and sphingomyelins (SMs) [198,199], although these patterns can vary between EV sources [200,201].

Several studies have shown that lipids can play roles in cargo transfer of EVs. Such functionalities result mostly from the fusogenic ability of specific lipids, allowing EVs to fuse with cellular membranes and release their cargo in recipient cells. One of the most striking examples of this phenomenon was described by Parolini et al, who used an R18-based fluorescence dequenching assay to show that EVs from melanoma cells can fuse with membranes of their parent cells in a pH-dependent fashion and could thereby transfer caveolin-1 to the membrane of recipient cells [202]. The authors suggested that this was mainly due to the lipids in the EVs, given that paraformaldehyde-fixated EVs showed largely preserved fusion capacity compared to untreated EVs, although they could not rule out a contribution of proteins in the pH-dependent fusion process. The importance of proteins in the fusion process was corroborated by others, who elegantly showed that treatment of EVs with proteinase K largely abolished release of their cargo when mixed with cellular membrane sheets at low pH [203].

4.4.1. Phosphatidylserine

In addition to proteins, cholesterol in cellular membranes was shown to contribute to EV membrane fusion as fusion was inhibited by the cholesterol-binding compound filipin [202], a finding also reported by others [204,205]. In addition, it has been shown that a mutant epidermal growth factor receptor (EGFRvIII) could be transferred from EV membranes to membranes of recipient cells, implicating fusion of EV membranes with those of recipient cells. Moreover, this transfer could be blocked by the PS-binding protein Annexin V, suggesting that PS is involved in such fusion processes or in the events preceding fusion [206,207]. Using similar methodology, PS exposure by EVs has been shown to be an important factor for EV binding and internalization by recipient cells in various EV-cell combinations (e.g. [120,121,204,208–211]). In addition, a recent study showed that fusion of R18-loaded liposomes with endosomal membranes in hepatocyte carcinoma (Huh7) cells depended on liposomal PS and cholesterol, but not on phosphatidylcholine (PC) [212]. Furthermore, PS-containing liposomes inhibited release of Hepatitis A virions from internalized PS-exposing EVs into the cytoplasm of recipient cells, suggesting a shared mechanism [212]. EV uptake by macrophages and monocytes could also be inhibited by PS- or phosphatidylglycerol (PG)-containing liposomes [119,209], in accordance with the presence of PS receptors such as TIM4 on such cells [213]. Despite these reports showing that PS is an important molecule in the EV membrane involved in cell binding and subsequent internalization, it remains to be investigated what role PS plays in cargo delivery. Fusion assays based on R18 dequenching can be confounded by other events than fusion, such as endosomal rupture or nonspecific probe transfer [108,214], and should therefore be executed with appropriate controls. Furthermore, it could be that the interaction of PS on EV surfaces with recipient cells is mostly relevant for phagocytic cells that express PS-receptors [213], but not for other cell types. Phagocytic cells normally use these receptors to scavenge and degrade PS-expressing apoptotic cells in a process termed efferocytosis [215,216]. Hence, the presence of PS in the EV membrane may drive such EVs towards clearance by macrophages (supporting their presumed role as cellular waste disposal systems) rather than functional cargo transfer. Indeed, a study in which the fate of gold nanoparticles encapsulated in lipid membranes were followed in cells revealed that high PS concentrations in the particle membranes resulted in high cellular uptake and subsequent particle accumulation in lysosomes, whereas lower PS concentrations resulted in accumulation in non-endolysosomal compartments instead [217]. These findings imply that PS indeed destines internalized particles for degradation rather than content transfer, though such effects may differ between cell and nanoparticle types [204,217].

4.4.2. Lysobisphosphatidic acid

Another EV membrane lipid that may be important in EV-mediated cargo transfer is the anionic lipid lysobisphosphatidic acid (LBPA), also known as bis-(monoacylglycerol)phosphate (BMP). Yao and coworkers showed that macrophage-derived EV uptake by hepatocytes was dependent on the interaction of EV-exposed PS with TIM1 on the hepatocytes [204]. Of note, this interaction was only found to be important for this combination of cell types, but not for other combinations. After internalization, EVs labelled with R18 showed dequenching at late endosomal (Rab7-positive) compartments, suggestive of fusion events. The dequenching signal colocalized with the late endosomal LBPA, and incubation of the cells with antibodies against LBPA resulted in increased accumulation of EV content in lysosomes, indicating compromised ability to escape the endosome [204]. This finding indicates that EVs may use similar LBPA-mediated endosomal escape mechanisms as phosphorothioate-modified antisense oligonucleotides [218] and viruses [219] to release their content in recipient cells. Of note, while it is known that specific viral proteins such as vesicular stomatitis virus (VSV)-G and Dengue protein E rely on LBPA for viral content release within recipient cells [220,221], the component in EVs that interacts with LBPA remains to be elucidated. Interestingly, LBPA is involved in the formation of intraluminal vesicles in MVBs during EV biogenesis, but is not incorporated in EVs itself (unless endosomal functional is perturbed [222,223]). It has recently been shown that the ESCRT protein Alix interacts with LBPA to sort cargo to EVs in this process

[224], and it is tempting to speculate that this interaction also takes place in the endosome after EV internalization.

4.4.3. Other lipids

Whereas multiple lipids, such as PS, SM, GM3 and cholesterol and are often found to be enriched in EVs compared to their parent cells, only the role of PS has been relatively well examined in the context of EV-cell interactions. For the other lipids, their role in EV cargo transfer remains unclear (we refer to [225] for an excellent review on the topic). It could be that their enrichment in EVs is the mere consequence of their contribution to EV biogenesis. For example, SM is enriched in lipid rafts and is involved in sorting of endoglin to EVs in the placenta [226], and inhibition of neutral sphingomyelinases generally disrupts EV secretion [227]. On the other hand, EV lipids may have signaling properties. For example, it has been shown that GM3 in EVs inhibits migratory behavior of recipient melanocytes, whereas its metabolite GD3 improved migration [228]. In addition, neuroblastoma-derived EVs may accelerate α-synuclein aggregation in Parkinson's disease [229]. Alternatively, when EVs are internalized by recipient cells in endosomes, similar lipids and proteins may be encountered as during EV biogenesis. During this process, lipids involved in EV biogenesis may again engage endosomal membranes to facilitate cargo transfer, or guide EVs towards specific subcellular locations as has been reported for GM3 and PS [217].

4.5. Engineered viral proteins: A toolset to boost EV-mediated functional cargo delivery

Despite the initial enthusiasm of the field for harnessing EVs for delivery of nucleic acids and proteins, it is increasingly becoming clear that the apparent rate by which these molecules (especially nucleic acids) are functionally transferred to recipient cells may not be as high as initially anticipated. This is most likely due to the low native encapsulation efficiencies of therapeutically attractive nucleic acids (e.g. miRNAs and mRNAs) in EVs by the EV-secreting cells. Current estimations state that most miRNAs and mRNAs are secreted in EVs at a disappointing rate of 1 molecule per tens to millions of EV particles [230–236], which is in stark contrast to the number of cytosolic and membrane protein copies in EVs which – depending on the type of protein - can range from tens to hundreds of copies per EV [237–240]. It has been suggested that these RNA species are not homogenously distributed over EVs, but clustered in specific subpopulations instead [241]. Despite this low relative abundance of RNAs, we and others have shown that EVs are able to functionally transfer RNAs to recipient cells [14,129,242]. However, several reports have shown that the natural ability by which EVs release cargo into the recipient cells' cytosol is limited by lysosomal degradation, a barrier also encountered by many synthetic drug delivery systems [243]. Viruses utilize specific molecules, in particular fusogenic proteins, to escape the endosome before such lysosomal degradation can occur. Moreover, evidence is emerging that some viruses use EVs as 'Trojan horses' to avoid immune recognition and facilitate entry into, and release from their target cells (as reviewed elsewhere [244,245]). In this process, viruses can modify the surface of EVs with viral proteins that can improve EV cargo transfer. For example, it has been shown that B-cells infected with Epstein-Barr virus (EBV) secrete EVs containing the viral envelope protein gp350, which promotes EV adhesion to B-cells rather than monocytes [246]. In a similar fashion, an envelope protein of the Zika virus (ZIKV-E) was detected on the EVs from infected mosquito C6/36 cells and these EVs could trigger inflammatory responses in endothelial vascular cells [247]. It was also recently shown that retroviral transduction, a common laboratory technique to genetically engineer cells, results in the secretion of EVs carrying the retroviral envelope protein syncytin-1. Through the fusogenic activity of this protein, the EVs could functionally transfer exogenous genes to recipient cells [248]. Syncytin-1 is also known to be endogenously incorporated into placenta-derived EVs and to convey immunosuppressive signals to the maternal immune system [249,250]. It has been reported that syncytin-1 and syncytin-2 mediate uptake of such EVs by recipient cells [251], and potentially facilitate EV fusion and concomitant cargo delivery [252]. Hence, for some viruses the incorporation of viral proteins in host-derived EVs is a naturally occurring process and may potentiate both EV and viral functionality.

The artificial enrichment of EVs with virus-derived proteins has also been explored as a way to improve the cargo delivery efficiency of EVs. A provocative recent pre-publication showed that engineered EVs containing CD63-β-lactamase fusion proteins were efficiently taken up by recipient cells (of the same cell type as the EV-secreting cells) loaded with a β-lactamase substrate, but failed to elicit substrate cleavage [109]. This was presumably because of the low endosomal escape efficiency of the EVs. When EVs were engineered to also contain the viral fusogenic protein VSV-G, known to incorporate into EV membranes upon cell transfection with VSV-G encoding plasmids [253], the β-lactamase substrate was cleaved efficiently upon EV uptake. Similar findings were reported for other combinations of EV donor and reporter cell lines, illustrating that EV mediated delivery of β-lactamase was critically dependent on the presence of VSV-G in the EV membrane [109]. In a similar fashion, fragments of a Nanoluc enzyme could only be delivered to the cytosol of recipient cells using EVs when the EVs were engineered with VSV-G [254]. In support of these reports, Gentili and coworkers showed that virus-like particles containing VSV-G could functionally transfer the second messenger cGAMP to recipient cells through fusion, whereas cGAMP-containing EVs derived from the same cells failed to do so [255], presumably due to the lack of fusogenic/endosomal escape capacity of the latter. These studies highlight that EVs' capacity to evade lysosomal endosomal degradation can be substantially improved by the incorporation of viral proteins. However, this may not always be the case, as Hung *et al* have shown that even in the presence of VSV-G, a substantial amount of EVs can be targeted for degradation in some EV-cell combinations [256]. Interestingly, also viral activity can be improved by their association with EV components. For example, a striking study of György and coworkers showed that adeno-associated virus (AAV) particles could be found within EVs and associated to their surface after ultracentrifugation of the medium of the vector-producing cells (a common EV isolation technique). EV-associated AAV outperformed 'naked' AAV in terms of transgene delivery efficiency both *in vitro* and *in vivo*, where more types of hair cells were transduced after administration via two different routes in the ear. Using this EV-AAV system the authors delivered Lhfpl5, a gene important for mechanotransduction in the ear, to Lhfpl5 knockout mice (displaying a deaf phenotype) and showed that hearing was largely restored [257].

Taken together, incorporation of viral components in EVs (and *vice versa*) can have synergistic effects for both EV and virus effectivity. Such combinations may have already evolved spontaneously (as in the case of syncytins in placenta-derived EVs), or may be artificially introduced to improve EV-mediated cargo delivery. However, it remains to be investigated to what extent such modifications are detrimental to the alleged immunoprivileged state of EVs, as viral proteins may provoke undesired immune recognition *in vivo* [258]. Furthermore, the stable expression of fusogenic proteins such as VSV-G in EV-producing cells can be problematic due to their cytotoxicity [259], which may limit reproducibility and manufacturability of the resulting engineered EVs. It could well be that also without such modifications, EVs contain some endogenous capacity to escape the endosome, provided that the right combination of EV donor and recipient cell type are studied. In fact,

Table 2
Overview of reporter systems to study EV uptake and cargo delivery with their strengths and limitations, as discussed in this review.

Strategy	Mechanism	Examples	Strengths	Limitations
Fluorescent hydrophobic dyes	Fluorescent lipid membrane labeling	PKH26, PKH67, DiD, DiI, DiO, DiIC.	Bright signal, easy staining protocols. Suitable for studying EV uptake.	Aggregate / micelle formation, membrane switching, particle size shift. Not suitable to study functional cargo delivery
Fluorescent self-quenching amphilipic dyes	Fluorescent lipid membrane labeling. Propagation of the probe stops quenching and activates fluorescent signal	MemBright	Efficient labeling, very bright signal, easy staining protocol. No observed effect on EV particle size. Suitable for studying EV uptake.	Not suitable to study functional cargo delivery
Fluorescent antibody labeling	Antibody-mediated fluorescent labeling of EV surface markers	Fluorophore-conjugated antibodies against tetraspanins (e.g. CD9, CD63 and CD81)	High specificity, allows for staining specific EV subpopulations. Suitable for studying EV uptake.	Not suitable for general staining of full EV population, antibodies may affect protein functionality and particle size. Not suitable to study functional cargo delivery
General fluorescent protein labeling	Maleimide-conjugated fluorophores, N-Hydroxy succinimidyl-conjugated fluorophores	Alexa-NHS, C_5-maleimide-Alexa	Bright signal, easy staining protocols. Suitable for studying EV uptake.	May affect membrane protein functionality, risk of protein aggregate staining, not suitable for only tracking intraluminal cargo. Not suitable to study functional cargo delivery
Membrane-permeable protein dyes	Treating isolated EVs with fluorescent cell permeable dyes	CFSE	Bright signal, easy staining protocols. Suitable for studying EV uptake.	May affect functionality of membrane proteins. Not suitable to study functional cargo delivery
Activatable membrane-permeable dyes	Treating cells or EVs with fluorescent membrane permeable dyes that are activated by intracellular components to become membrane impermeable	Calcein-AM, Calcein Violet	No interfering fluorescent signal from stained protein aggregates. Suitable for studying EV uptake.	Membrane permeabilization post-staining results in loss of signal due to leakage. Not suitable to study functional cargo delivery
Activatable membrane-permeable protein dyes	Treating cells or EVs with fluorescent membrane permeable protein dyes that are activated by intracellular components and covalently attach to intracellular amines	CFDA-SE	No interfering fluorescent signal from stained protein aggregates. Suitable for studying EV uptake.	Not suitable to study functional cargo delivery
Fluorescent fusion proteins	Cellular expression of EV-associated proteins fused to fluorescent proteins	Fusion of eGFP/RFP to tetraspanins CD9, CD63, CD81	No interference with membrane protein functionality, high specificity, allows for staining specific EV subpopulations. Suitable for studying EV uptake.	Not suitable for general staining of full EV population, not suitable to study functional cargo delivery
Post-translationally modified fluorescent proteins	Cellular expression of fluorescent proteins with post-translational modifications for membrane incorporation	Fusion of eGFP/RFP with myristoylation or palmitoylation sequences	Allows for a more homogenous, general labeling of EVs with fluorescent proteins. Suitable for studying EV uptake.	Distribution in EV subpopulations not yet fully characterized. Not suitable to study functional cargo delivery
Metabolic labeling	Incorporation of metabolic precursors into glycoproteins, which are decorated with fluorescent conjugates	Strain-promoted azide-alkyne click (SPAAC) chemistry	High specificity, low toxicity, no micelle formation, potential versatility in conjugate functionality, easy post-isolation EV modification.	Distribution in EV subpopulations not yet fully characterized. Effect on EV membrane protein functionality / EV biodistribution currently unknown.
Prion transfer	Transferring misfolded prions to cell expressing eGFP-labeled prions in their native conformation.	*S. cerevisiae* Sup35 prion transfer	Suitable for studying functional protein delivery	Effects of misfolded protein structure on EV transfer currently unknown.
Luciferase	Cellular expression of EV-associated proteins fused to luciferases.	Renilla luciferase, firefly luciferase, NanoLuc, ThermoLuc	Luciferase is suitable for high-throughput sensitive readout. Suitable for studying EV uptake.	Luciferase readout does not discriminate between membrane-bound, cytosolic, or endo/lysosomal localization.
Split luciferase	EV-mediated delivery of a luciferase fragment to a target cell with cytosolic expression of the complementary part of luciferase.	Split Renilla Luciferase	Luciferase is suitable for high-throughput sensitive readout. Non-cytosolic luciferase fragments should be inactive. Suitable for studying EV uptake.	As luciferase read-out often requires cell lysis, contamination with separate cellular compartments is a potential risk.
Split fluorescent proteins	EV-mediated delivery of a fluorescent protein fragment to a target cell with localized expression of the complementary part of the fluorescent protein.	Split eGFP	No cell lysis is required for analysis. Compatible with live-imaging, and can be used to study specific subcellular compartments. Suitable for studying EV uptake.	Subcellular compartments with low pH may interfere with signal in live imaging.
miRNA/siRNA transfer targeting endogenous targets	miRNA/siRNA transfer targeting endogenous genes or pathways.	Measuring expression levels of target genes, or studying phenotypic behavior like migration/ proliferation.	Representative assay for intrinsic EV-mediated intercellular communication. Suitable for studying functional RNA delivery.	Low sensitivity due to variation of target expression levels in bulk cell population. EVs may contain additional components that may affect readout
miRNA/siRNA transfer targeting non-endogenous targets	miRNA/siRNA transfer targeting of non-endogenous reporter constructs.	Targeting fluorescent proteins or luciferase	Suitable for studying functional RNA delivery. Low chance of interference due to indirect or off-target effects.	Low sensitivity due to variation of target expression levels in bulk cell population.

(*continued on next page*)

Table 2 (*continued*)

Strategy	Mechanism	Examples	Strengths	Limitations
mRNA transfer	Transfer of mRNA molecules that are translated to protein in target cells	Cre recombinase to activate LoxP-flanked fluorescent reporters, transfer of Gaussia Luciferase mRNA	Compatible with common *in vitro* and *in vivo* reporter systems. Single-cell accuracy at high sensitivity. Low chance of interference due to indirect or off-target effects. Suitable for studying functional EV cargo transfer.	Low abundance of full-length mRNA in EVs. No straightforward protocols to discriminate between reporter activation due to transfer of mRNA or translated protein.
sgRNA transfer	Transfer of sgRNA to cells expressing Cas9	CROSS-Fire reporter system	Suitable for studying functional RNA delivery. Single-cell accuracy at high sensitivity. Low chance of interference due to indirect or off-target effects. Specific for RNA transfer, as RNA translation is not required for reporter activation.	Loading mechanisms of sgRNA as compared to endogenous RNA not yet characterized.
Labeling endogenously expressed RNA	Expressing fluorescent proteins with RNA-binding sequences in the 3′-UTR	MS2/PP7 binding sequences	Suitable for visualizing RNA uptake and intracellular trafficking and processing. Specific for modified RNA.	No functional read-out for RNA delivery.

our group recently showed that EVs derived from two different cancer cell lines were more efficient at functionally delivering sgRNA to HEK293T recipient cells than current state-of-the-art Dlin-MC3-DMA-based lipid nanoparticles [260]. It would be valuable to study endosomal escape mechanisms in cell types in which functional EV content transfer is observed, given that the molecular mechanisms for EV-mediated endosomal escape remain unclear. In this regard, the use of primary cells or animal models may give more relevant information on EV-mediated cargo transfer than the use of immortalized cell lines. However, the technical difficulties associated with the former systems in combination with the challenges associated with large-scale EV production (and potential engineering) have so far resulted in limited insight in the mechanisms by which EVs can fuse with cellular membranes to promote cargo delivery [261].

Functional EV cargo transfer is usually studied using specialized cellular reporter systems. These reporter systems differ in terms of sensitivity, specificity and tendency to be biased by confounding factors. The information derived from such reporter systems form the basis of our current understanding of the mechanisms by which EVs functionally deliver their cargo to recipient cells. In the following sections, we will discuss the types of reporter systems for EV cargo transfer that have been applied so far and their advantages and disadvantages. In addition, we will highlight the information that such systems have yielded regarding cellular mechanisms involved in functional EV cargo transfer.

5. Reporter systems to study EV cargo transfer

The discovery of EV-mediated RNA transfer by Valadi et al in 2007 sparked a high interest in the unraveling of the mechanisms that dictate EV uptake, trafficking, and intraluminal cargo delivery [14]. As a result, the last decade has seen a vast and constant development of novel reporter assays and techniques to study these processes. These tools have helped to clarify the processes that regulate EV-mediated cargo delivery. However due to the novelty of the EV-field, as well as the technical limitations of many commonly used reporter systems, there remains much to be elucidated. In this section, we will provide an overview of the cellular reporter systems that have been developed to study EV-mediated cargo transfer. These reporter systems are summarized in Table 2.

5.1. Fluorescent labeling of EVs

The most commonly used method to study the kinetics and regulatory processes of EV uptake is by tracking transfer and uptake of fluorescently labeled EVs. The easiest method of fluorescently labeling EVs is through the application of hydrophobic dyes after EV isolation. Commonly used labels include PKH26 and PKH67 (red and green fluorescent, respectively) and carbocyane dyes such as DiD, DiI, DiO and DiIC dyes [42,262–264]. As these lipophilic dyes efficiently label EVs and emit bright fluorescent signals, they are used to study EV uptake kinetics and pathways using flow cytometry and fluorescence microscopy. Alternative membrane dyes that are regularly used include boron-dipyrromethene-labelled lipids or rhodamine-labelled lipids [265,266]. Studies in which EVs have been labeled with such dyes have been instrumental in unraveling the main pathways through which EVs are taken up by cells. However, use of such dyes does require caution, as they have been shown to come with certain drawbacks. Lipophilic dyes such as PKH and carbocyane dyes may form aggregates or micelles in the aqueous conditions in which EVs are commonly suspended, which may obscure sample particle quantification and interfere with EV uptake analysis as the fluorescent signal from these particles is hard to distinguish from that of labeled EVs [267]. Moreover, unbound dye in the sample may immediately stain cellular membranes [268]. As such, appropriate washing protocols and control conditions are essential when using these dyes. Secondly, non-specific dye leakage, which may result in passive dye switching between membranes, is a risk with these hydrophobic, non-covalent dyes. Lastly, it has been reported that labeling EVs with such dyes can result in increased particle sizes [269], which may affect cellular uptake. A recently developed self-quenching cyanine-based probe with amphiphilic anchors, called MemBright [270], is an interesting novel alternative that ameliorates some of these issues. As MemBright self-quenches, aggregates of this probe do not emit a fluorescent signal. However, upon integration in the EV membrane the probe propagates and starts emitting a bright fluorescent signal. In a comparison with PKH, MemBright labeling resulted in a higher brightness and MemBright labeling did not affect EV particle size [271].

Similarly, EVs can also be labeled post-isolation by fluorescently labeled proteins. Such strategies may include the use of fluorescent antibodies that target EV-associated proteins [264]. This approach allows for the tracking of specific subpopulations of EVs based on their surface protein profile. However, the presence of these antibodies may affect EV uptake by influencing particle size, or by interfering with receptor-ligand interactions by blocking physical interaction with specific protein domains. A more general staining approach is the use of maleimide-conjugated fluorophores, that bind to thiol groups of cysteine-rich domains [272]. This approach was shown to have no significant effect on EV size distribution. A similar approach is through the use of Alexa Fluor N-Hydroxy succinimidyl (NHS), a fluorophore bound to an NHS ester, which forms

covalent bonds with amine groups [273]. Whereas the effect of this labeling method on EV functionality and biodistribution has not been extensively studied, there is a potential risk of affecting these processes through the use of NHS-esters to covalently bind fluorophores to amine groups on the outside of EVs, as EV membrane proteins play a pivotal role in these processes. As such labels may also stain protein aggregates or soluble proteins still present in the sample, use of such dyes also requires appropriate washing protocols.

Additionally, EVs are frequently fluorescently labeled using membrane-permeable dyes, such as CFSE, a membrane-permeable molecule that contains a succinimidyl group [274]. The cell-permeable compound calcein-acetoxymethyl (Calcein-AM) is an interesting alternative as, unlike CFSE, it is not fluorescently active in its native extracellular state [275]. Calcein-AM is processed by intercellular esterase activity, cleaving off the acetoxymethyl group to form a water-soluble fluorescent molecule. As such, this dye may be administered to EV-producing cells, to subsequently end up in its fluorescent form in EVs. A combination of the aforementioned approaches is the use of membrane-permeable protein dyes that have been specifically designed to label intraluminal proteins. For example, CFDA-SE (occasionally confused with CFSE) is a cell permeable diacetate precursor of CFSE, and has higher cell permeability. After uptake, intracellular esterases remove the acetate groups resulting in the formation of the less cell permeable fluorescent molecule CFSE [276]. CellTracker dyes such as Cell Tracker Deep Red have similar functionality [177]. As these dyes rely on an enzymatic conversion of a non-fluorescent dye to a fluorescent dye after being taken up by EVs, these dyes show a significant decrease in non-specific labeling of protein aggregates.

Lastly, a commonly employed method to fluorescently label EVs is through the expression of fluorescent proteins fused to EV-associated proteins [262,267,277]. Fluorescent proteins like eGFP, or red fluorescent proteins such as RFP, mCherry or TdTomato are regularly used, as they can easily be analyzed using flow cytometry and fluorescence microscopy alongside other fluorescent probes [278]. Expressing fluorescent proteins in their unmodified soluble state may also result in some stochastic loading into EVs [279], however this occurs at low levels. The most frequently used EV protein anchors for fusion with fluorescent proteins are the tetraspanins CD9, CD63, and CD81, which are commonly used EV markers [280]. As both the C- and N-terminus of these transmembrane proteins are intraluminal, fluorescent proteins have been attached on both termini of these tetraspanins. Additionally, such strategies may be combined with pH-sensitive fluorescent proteins like pHluorin [281]. As these fluorescent proteins are quenched in the low pH environment of the late endosome, fusion events of the MVB with the cell membrane may be visualized due to the rapid rise in pH and subsequent unquenching of the fluorescent proteins [282]. An advantage of fluorescently labeling EVs using fusion proteins is the possibility to separately study the uptake of specific EV subpopulations, based on their proteomic content. However, it is also possible to achieve a more general EV labeling strategy by fusing fluorescent proteins with post-translational sequences to induce membrane association. Examples are the covalent attachment of fatty acids by a palmitoylation sequence [283], a myristoyl group by a myristoylation sequence [284], or esterification by an acetylation sequence [285]. As fluorescent proteins are generally fused to EV-associated proteins, there is a substantially decreased risk of fluorescently labelled protein aggregates as compared to post-isolation labeling strategies. Moreover, EVs may be used directly upon isolation, as no additional yield-limiting staining or washing steps are required. However, this approach requires either the use of transient transfection, or the generation of stably expressing cell lines with such constructs. As commonly used transfection reagents are known to cause cell stress, affecting membrane integrity and cellular behavior and end up in EV preparations [286], the generation of stable cell lines is preferred. However, this may be challenging for primary cells. Lastly, some caution should be taken in case of live cell imaging, as the low pH of the late endosomal pathway may affect fluorescence of certain fluorescent proteins [287].

Another efficient, yet less commonly used approach to track EVs, is through metabolic labeling. This approach is based on the use of biorthogonal chemistry to label EVs. An advantage of this approach is the potential for high specificity and low toxicity [288]. Unnatural metabolic precursors such as azide containing sugars can be administered to EV-producing cells and can then be subsequently incorporated into glycoproteins through the natural glycosylation pathway [289]. Using click chemistry approaches, these azide-containing sugars can then be targeted by reactive groups-containing fluorescent dyes. As the azide-containing sugars are present on the cell membrane, they can also be found on the membrane of EVs. For example, Lee et al. used copper-free and strain-promoted azide-alkyne click (SPAAC) chemistry, resulting in azide-decorated extracellular vesicles that were then targeted by azadibenzylcyclooctyne-containing fluorescent dyes [290]. EVs labeled with this technique were successfully used to track both *in vitro* cell uptake and *in vivo* biodistribution. Wang et al. use a residue-specific labeling strategy based on the incorporation of non-canonical amino acids into proteins synthesized by the cell: L-azidohomoalanine (AHA), an azide-bearing amino acid analogue of methionine. They also cultured EV-producing cells with an azidosugar that was metabolized into sialic acid. The azides were then targeted by dibenzobicyclooctyne (DBCO) using click chemistry to label the EVs with Cy3, or indirectly with FITC [291]. Alberti et al. incubated cells with Ac4ManNAz for 72 h and stained EVs using cyclooctyne-modified fluorescein isothiocyanate (ADIBO-FITC) to demonstrate *in vitro* cellular uptake [292]. These approaches have substantial benefits: cells are not exposed to and affected by transfection reagents, and as the conjugates used for click chemistry do not form micelles, unbound dye is easily separated from EVs. They also offer great versatility in the types of conjugates that can be used to decorate the EVs post-isolation, enabling a range of applications. However, as mentioned with certain covalent protein dyes, some caution is recommended as the effects of coating EVs with these conjugates on EV functionality and biodistribution has not yet been extensively elucidated.

5.2. Reporters for functional protein delivery

Fluorescent labeling has been a highly valuable tool for the study of EV uptake and intracellular trafficking. However, such strategies may give limited information on intracellular cargo release and functional delivery. To gain a better understanding of the regulation of these processes, there has been an increased focus on development of assays focused on intracellular functionality as well as cellular localization of EV cargo. As it is currently not known whether these processes differ for EV-mediated delivery of proteins and nucleotides, separate assays are being employed to study these cargoes. Firstly, there are read-outs based on phenotypical changes in cell behavior due to transfer of endogenously expressed proteins, such as migration, proliferation, or protein expression. Whereas such read-outs represent EVs' natural functionality and therapeutic potential [293], they are generally not suitable to study specific cargo transfer and processing pathways [254]. Firstly, it is possible that EV-associated factors may interact with target cells via receptor-ligand interactions at the cell membrane prior to EV uptake [294]. Secondly, these processes may be

affected by a multitude of proteins and RNAs present in EVs [295,296]. Moreover, changing expression of one specific factor in EV-producing cells may affect expression of other proteins and RNAs, resulting in additional changes in EV functionality and its effect on the target cell's phenotype. Thus, tracking of non-endogenously expressed cargo may be preferable. One such strategy, is through the use of prions. Prions are misfolded proteins that transfer their misfolded shape to normally folded variants of that same protein [297]. Prions are transferred between cells through various pathways, including EVs. In an elegant study by S. Liu *et al.* EV-mediated prion transfer was shown using fluorescent proteins [298]. Reporter cells expressed GFP-tagged cytosolic proteins in their properly folded formation, and were shown to aggregate through fluorescence microscopy within 12 h after addition of EVs isolated from prion-infected cells. This assay allows the study of EV-mediated functional transfer at single-cell accuracy. However, it should be noted that it is not known whether EV-mediated transfer of misfolded proteins occurs in a similar manner as that of properly folded proteins. An additional approach is the functional measurement of EV-mediated transferred proteins with enzymatic activity. Transfer of luciferases is a sensitive, straightforward approach that has been employed to study *in vivo* EV biodistribution, as well as EV uptake kinetics [299,300]. A drawback of *in vitro* luciferase-based analyses for EV uptake is that cell lysis is generally required before substrate addition. As such, it is not possible to determine whether the luciferase was indeed present in the cytosol, whether it was still encapsulated in EVs present on the cell surface, or whether it was retained within the endolysosomal pathway. To address this issue, split proteins may be employed. In this approach, a protein is split in to two non-functional parts, which are able to reassociate upon mixing [301]. By fusing one part of the split protein to an EV-associated protein of a donor cell, and expressing the additional part in the cytosol of a target cell, one can study EV cargo delivery through Western Blot analysis or enzymatic activity. One such example is the use of split luciferase as a sensitive, quantitative assay to study EV uptake [302]. However, as cell lysis is still often required for luciferase activity measurements some caution should be taken with this approach, since this could result in the release of the content of intracellular compartments. A notable exception is a recent report of M. Somiya and S. Kuroda, who demonstrate intracellular luminescence imaging of Nanoluc luciferase through luminescence microscopy [303]. Use of split fluorescent proteins, such as eGFP, does not require cell lysis and, although it may be less sensitive than enzymatic interactions such as luciferase activity, it does allow for single-cell analysis using flow cytometry and intracellular tracking using fluorescence microscopy [302]. Additionally, fusing fluorescent split protein to proteins associated with specific cellular compartments [304] holds the theoretical potential to provide additional information on post-uptake intracellular cargo trafficking.

5.3. Reporters for functional RNA delivery

In order for RNA to exert its function, it needs to be delivered to the cytosol of the target cell. Thus, as previously mentioned, use of fluorescently labeled EVs is not suitable to study pathways for RNA transfer, as these assays do not provide information on functional cargo delivery. Similar to studying protein delivery, there are readouts for functional RNA transfer that rely on phenotypical changes in cell behavior or gene expression. However, as EVs contain a large number of molecules that may affect such processes, these readouts are neither sufficiently sensitive nor specific to fully demonstrate and study RNA transfer [254]. As such, targeting or delivery of non-endogenous constructs are preferable, such as RNAs targeting fluorescent proteins or luciferase [78]. Transfer of miRNA/siRNA against luciferase is an assay with a high-throughput readout potential for analysis of bulk cell populations. The advantage of this approach is its specificity, as the chance of off-target effects can be minimized by rational design of the RNA sequence. However, small effects on expression levels (or strong effects on a small number of cells) may be masked by expression levels of the total cell population, affecting the robustness and sensitivity of this assay. Luciferase signal could also be affected by general effects of EVs on cell proliferation, senescence, or metabolism [305]. These effects could be corrected for using dual luciferase reporter constructs. Such constructs could even be employed to study transfer of endogenous miRNAs, by incorporating targeting sequences in the 3′ UTR of a luciferase open reading frame [306]. Targeting fluorescent proteins addresses the issue with total cell population expression levels, as this allows for single-cell analysis. However, variation in expression levels within the general cell population, as well as the long half-life of fluorescent proteins strongly affect sensitivity of such assays. This can be partially resolved by using unstable fluorescent protein variants, so that knockdown effects can be detected more rapidly [307]. As positive readouts are generally more sensitive than negative (e.g. knockdown) readouts, various assays have been developed to induce expression of non-endogenous proteins instead, such as the EV-mediated transfer of mRNA for Gaussia Luciferase [283]. An intrinsic limitation of such mRNA-based strategies is the potential transfer of the encoded protein, translated in the EV-producing cell, alongside the mRNA [254]. As these systems do not fully differentiate between RNA and protein delivery, assessing protein levels or functionality within the EV population is required. In the case of Gaussia Luciferase however, as it is secreted in soluble form, we observed no substantial luciferase activity in EVs [129]. A popular mRNA-based reporter system with single-cell accuracy is the Cre-LoxP system [308]. In this system, Cre recombinase is expressed in donor cells, and co-cultured with cells expressing a fluorescent reporter protein flanked by LoxP sites [309]. Upon transfer of Cre mRNA, fluorescent protein expression is permanently activated in the target cell. As Cre-LoxP constructs are commonly used in *in vitro* and *in vivo* systems, this system has successfully been employed to demonstrate transfer of Cre in both *in vitro* and *in vivo* settings, both with and without direct cell contact [308]. Whereas this approach is generally considered a read-out for mRNA transfer, transfer of Cre protein is theoretically possible. Different studies have reported either a presence [310] or a lack of the Cre recombinase protein in EVs [308]. Recently, we generated the CRISPR operated stoplight system for functional intercellular RNA exchange (CROSS-FIRE), a CRISPR-Cas9-based reporter system that allows direct functional study of EV-mediated transfer of small non-coding RNA molecules at single-cell resolution [129]. In this system, donor cells express a single guide RNA (sgRNA), which targets a fluorescent reporter construct that, alongside Cas9, is expressed in reporter cells. After functional sgRNA delivery, Cas9 is able to generate a double-stranded break in the fluorescent reporter construct sequence. This can subsequently result in a 1 or 2 nucleotide frameshift within the reporter construct, both leading to permanent eGFP expression. Using siRNAs targeting specific genes involved in EV biogenesis in donor cells, as well as genes involved in endocytosis and intracellular membrane trafficking in reporter cells, we showed that this system is suitable to study the role of individual genetic targets in EV-mediated RNA transfer. As sgRNAs do not rely on translation to exert their functionality [311], this system specifically demonstrates functional delivery of RNA. However, one limitation of this system is the reliance on a non-endogenous RNA species, for which loading mechanisms – especially as compared to endogenous RNA - are not yet fully characterized. Lastly, RNA transfer may be visualized by incorporation

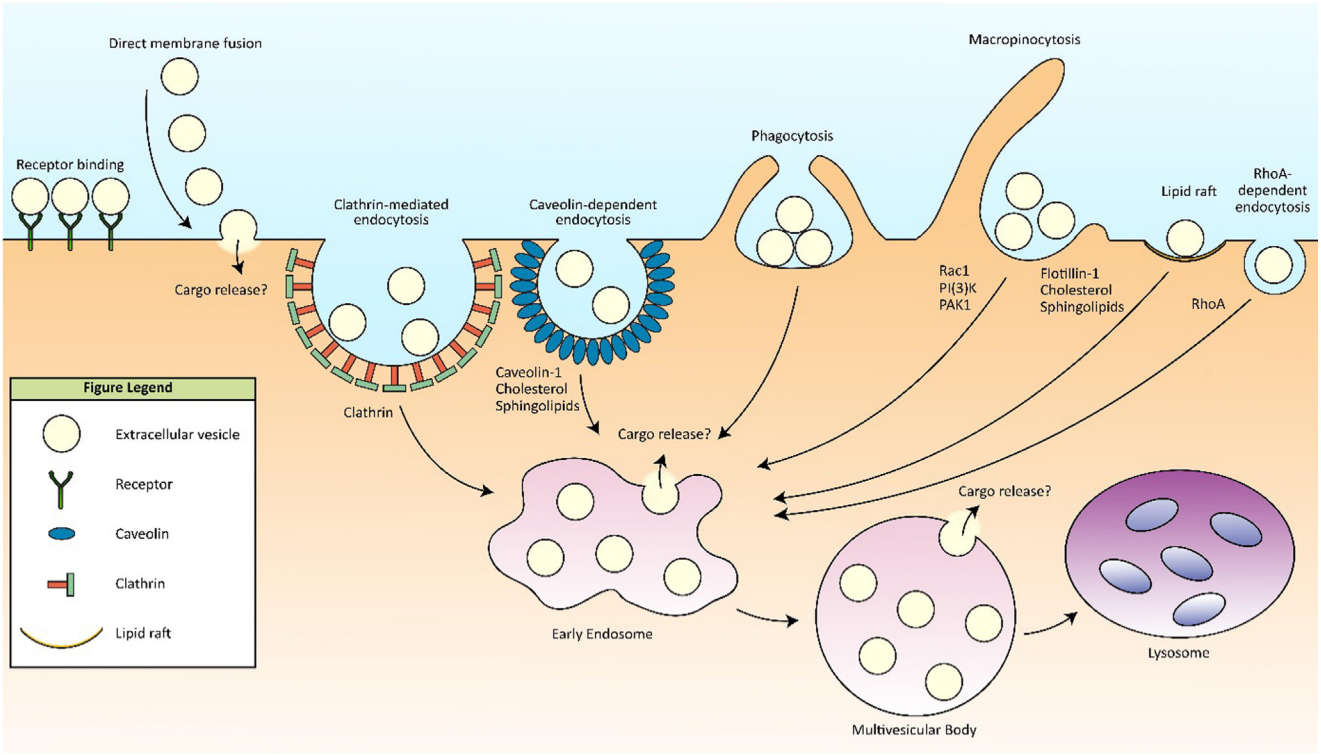

Fig. 2. Overview of mechanisms and regulating factors discussed in this review that regulate extracellular vesicle-mediated signaling and uptake in target recipient cells. EVs may interact directly with receptors on the cell membrane resulting in intracellular signaling cascades, or may deliver their intraluminal cargo into the recipient cell's cytosol through various mechanisms. EV cargo can be released to the cytosol through direct fusion with the cell membrane, for which there is currently limited evidence, or can be released after fusion with endosomal membranes after internalization. EV internalization occurs through endocytosis, which occurs through various mechanisms displayed in this figure. Trafficking of EVs to the lysosome through the endosomal pathway results in the degradation of EVs, and recycling of their contents.

of multiple RNA-binding sequences in the 3′UTR of mRNA molecules, such as MS2 binding sequences [283]. Fluorescent proteins fused to the MS2 coat protein can be expressed either in the EV-producing or recipient cell to allow fluorescence microscopy-based intracellular tracking of the RNA molecule. Whereas this approach does not report functional transfer of RNA, it can be used to visualize intracellular trafficking and processing of RNA transferred via EVs.

6. Cellular processes involved in EV cargo transfer

Alongside direct receptor-ligand interaction at the cell membrane, EVs also play a role in intercellular communication through the delivery of their intraluminal cargo. This cargo, which naturally consists of RNA molecules and soluble proteins, needs to be released from EVs into the cytosol upon delivery to be able to exert its function. For this to occur, a fusion event between the EV membrane and a membrane in contact with the cytosol of the target cell is required. This could occur directly at the cell membrane, or after active uptake of the EVs through endocytosis [91], as is shown in Fig. 2. If EVs are taken up through endocytosis, the cargo of the EV has to escape the endo-lysosomal pathway, in order to prevent lysosomal degradation. This process of endo-lysosomal escape has been reported to be the limiting factor for lipid nanoparticle-mediated RNA delivery [312]. Thus, direct fusion of EVs at the cell membrane would hypothetically be the most efficient method of cargo transfer. However, even though it has been shown that EV membranes in principle could fuse directly with the cell membrane, definitive evidence of EV cargo delivery through is process is, as of yet, limited [313]. There is however a substantial body of evidence that shows EV uptake through endocytosis using fluorescently labeled EVs. This is supported by the observation that fluorescently labeled EVs are not taken up at 4 °C, indicating that EV uptake is an energy-dependent active process [145,147]. Moreover, chemical inhibition of various endocytosis pathways has been shown to inhibit uptake of fluorescently labeled EVs, as well as EV-mediated transfer of RNA and protein [129,145,302].

6.1. Endocytosis

There are various mechanisms through which endocytosis may occur. Generally, these processes are sub-divided in phagocytosis and pinocytosis. Phagocytosis is generally involved in the uptake of opsonized particles that are larger than most EVs [313]. Phagocytosis-mediated uptake of tumor-derived EVs has been observed in macrophages, but as this was not observed in other cell types this does not seem to be a general mechanism for EV uptake [213]. Rather, EV uptake generally appears to be pinocytosis-mediated. There are multiple mechanisms of pinocytosis, including clathrin-dependent endocytosis, caveolin-dependent endocytosis, and macropinocytosis [314]. All these pathways have been demonstrated to play a role in EV uptake, and may contribute to a different extent to release of the EV cargo. Moreover, the occurrence as well as the effect on cargo delivery of these pathways may vary between cell types.

6.2. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) occurs through the assembly of clathrin-coated endocytic vesicles. Formation of these clathrin-coated vesicles results in the deformation of the cell mem-

brane, which ultimately leads to internal budding. After the clathrin-coated vesicle is pinched off, clathrin is removed and the formed vesicle then fuses with the endosome [314]. Conflicting data has been reported on the role of CME in EV uptake in various cell types. For example, Escrevente *et al.* reported a decrease in EV uptake when inhibiting CME using Chlorpromazine in SKOV3 cells [315], and CME was also shown to play a role in EV uptake in mesenchymal stem cells by Tian *et al.* [316]. However, Vader *et al.* observed an increased EV uptake in HeLa cells treated with Chlorpromazine, and no effect on EV uptake when knocking down clathrin heavy chain expression [145]. In contrast, transferrin uptake was strongly inhibited by Chlorpromazine. Feng et al. reported some effect of Chlorpromazine on EV uptake in macrophages but, somewhat in line with the observation in HeLa cells, this effect was minor as compared to the effect on transferrin uptake in macrophages [213].

6.3. Clathrin-independent endocytosis

Clathrin-independent endocytosis (CIE) pathways have also been shown to play a role in EV uptake, as well as functional cargo transfer [129,145,261,302,313,317]. One such endocytic pathway is caveolin-dependent endocytosis (CDE) in which caveolae, lipid rafts in the cell membrane enriched in sphingolipids, cholesterol and caveolins, are internalized [318]. Knockdown of Caveolin-1, a protein required for formation of caveolae, has been shown to strongly reduce EV uptake [145,319]. However, Caveolin-1 can also affect EV uptake indirectly, as Svensson et al. reported that Caveolin-1 negatively regulates EV uptake via ERK1/2 signaling [320]. Our group has shown that Caveolin-1 knockdown strongly inhibits RNA transfer both in direct co-culture in both HEK293T and MCF-7 cells and after EV addition in HEK293T cells using the CROSS-FIRE reporter system [129]. Caveolins are not the only components of lipid rafts that play a role in EV uptake. Flotillins, caveolae-associated integral membrane proteins, associate with lipid rafts and play a role in EV uptake as well [313,318]. Knockdown of Flotillin-1, coincidentally also commonly employed as an EV marker, also results in a decrease in EV uptake in HeLa cells [145]. However, unlike Caveolin-1, knockdown of Flotillin-1 in HEK293T and MCF-7 cells did not result in a decrease of RNA transfer using the CROSS-FIRE system [129]. Whereas it is thus tempting to speculate that EVs taken up through Flotillin-1-mediated mechanisms may play less important roles in RNA transfer than those taken up through Caveolin-1, uptake and RNA transfer experiments were done in different cell types and additional studies are required to draw such conclusions. An additional observation indicating a role for lipid rafts in EV uptake, is that of cholesterol. Indeed, inhibition of cholesterol synthesis using various compounds results in the disruption of EV uptake [145,213,320]. Alongside CDE and flotillin-dependent endocytosis, CIE can also be categorized into Arf-6, CDC42, and RhoA-dependent endocytosis [318]. Vader et al demonstrated that knockdown of Arf-6 in HeLa cells did not significantly affect EV uptake [145]. Rho kinases CDC42 and RhoA were also studied for EV uptake and RNA delivery in recipient cells. Interestingly, whereas knocking down RhoA results in a decrease in EV uptake and EV-mediated cargo transfer, knockdown of CDC42 does not [129,145]. Moreover, knockdown and chemical inhibition of Rho kinase effector ROCK1 also disrupts RNA transfer [129]. It should be noted that some caution is required when studying the role of Rho kinases in EV uptake, as they are involved in the regulation of numerous processes which potentially indirectly affect EV uptake. Unlike CDC42 and RhoA, Rho kinase Rac1 is involved in the regulation of macropinocytosis [321].

6.4. Macropinocytosis

Macropinocytosis is a process involving the uptake of large quantities of extracellular fluid through the formation of invaginated membrane ruffles. In this process an extension from the cell membrane protrudes from the cell and engulfs a portion of extracellular fluid, which is subsequently internalized due to the fusion of the membrane protrusion with the cell membrane [314]. This process has also been reported to result in EV uptake. Macropinocytosis is regulated by Rac1 and PI(3)K, and chemical inhibition of either of these proteins, through EIPA and wortmannin respectively, has been reported to decrease EV uptake in HeLa cells [145] and oligodendrocytes [322]. Macropinocytosis can be inhibited using Na+/H+ exchanger activity inhibitor EIPA [313]. EIPA inhibits EV uptake in oligodendrocytes, HeLa cells, PC12 cells, and BxPC-3 cells [145,172,316,322]. Macropinocytosis also plays a role in HEK293T cells, MCF-7 cells, and mesenchymal stem cells [129,316]. However, this could be cell dependent as no role was found for macropinocytosis in EV uptake in macrophages or Mantle cell lymphoma cells [213,323]. Similarly, whereas macropinocytosis regulator PAK1 knockdown results in decreased EV uptake in HeLa cells and RNA delivery in HEK293T cells, no effect was observed in MCF-7 cells, and whereas ANKFY1 knockdown has no effect on EV uptake in HeLa cells and RNA delivery in HEK293T cells, ANKFY1 knockdown increased RNA transfer in MCF-7 cells [129,145].

Taken together, these data strongly suggest that endocytosis plays an essential role EV-mediated transfer of intraluminal cargo, as disruption of multiple endocytic processes results in a decrease in EV uptake, as well as functional protein and RNA transfer. However, different studies show conflicting results in the role of the individual mechanisms and regulators. This difference may in part be explained due to the difference in used culturing conditions, reporter cell lines, EV-producing donor lines, and variation in EV isolation protocols. Furthermore, one should be more cautious when using chemical inhibitors as compared to targeted gene knockdown due to their potential non-specificity. An additional challenge in unraveling the role of specific endocytic mechanisms is the potential effect of cross-regulation [318], as inhibition of one specific mechanism may result in the increased activity of an alternative mechanism to compensate. Additionally, mechanistic studies on uptake and processing mechanisms of specific EV subpopulations are currently lacking. Therefore, it is currently not known whether all EV subpopulations are processed through similar mechanisms. Altogether, the exact moment and mechanisms of endosomal escape of EV cargo into the cytosolic compartment remain poorly understood. Currently, most strategies to increase cytosolic cargo delivery are based on the incorporation of viral proteins, as is described in Section 4.5. An exception is the use of connexins, gap junction proteins that are capable of forming hexameric channels that aid in the release of EV cargo into the cytosol [324,325]). Lastly, EVs may employ different mechanisms for functional delivery of proteins, RNA molecules and small compounds, which largely remain to be elucidated. However, with the development of increasingly sensitive and specific reporter systems for functional delivery of proteins and RNA molecules, these processes may finally be unraveled in the coming years, which may pave the road for tailored EV-based drug delivery systems.

7. Perspectives

As highlighted in this review, the EV research field has substantially grown over the past two decades, fueled by reports on the exceptional effects of EVs in different areas of biology and therapy. Along with the growth of the field, the conception that EVs are

more than cellular waste disposal systems has matured. However, despite the extensive research on EV biology, it remains unclear *how* EVs exert their effects and deliver their cargo. This topic remains particularly difficult to investigate, given that EV isolation protocols differ between research facilities and EV yields are generally insufficient for multiple experiments with the same isolated batch. In addition, the functional effects of EVs on recipient cells can differ between EV-secreting cell types and recipient cell types. To further complicate matters, EVs secreted from a single cell source are highly heterogeneous. This heterogeneity is reflected in the surface signature of EVs, but also in their cargo. For example, it has been shown that EVs immunoprecipitated with antibodies against different surface markers differ in their RNA and protein content [89,326]. These and other studies [327] suggest that there is a link between surface signature and EV cargo. It is conceivable that EVs' capacity to functionally transfer cargo is determined by this interplay, and that multiple functionalities can be ascribed to a single preparation of EVs [17,254]. For example, some EVs (potentially loaded with cellular waste products) may be destined for degradation by their surface signature, whereas others may be equipped with the molecules to bypass the endosomal-lysosomal pathway and release their content (such as second messengers or RNA species) in recipient cells. Indeed, it was recently elegantly shown that only a small fraction of EVs internalized by cells actually released their cargo in the recipient cell's cytosol [108]. In order to exploit EVs or EV components for drug delivery purposes, it is critical to identify what features are carried by such 'active' EV subpopulations, so that these EVs can be enriched or specifically engineered with therapeutic cargo. Hence, it appears critical that EV functionality is researched on a single particle or single EV subpopulation level to elucidate which molecules on EVs and recipient cells facilitate EV cargo delivery. Such molecules can provide important leads to improve existing drug delivery systems, for example by targeting nanoparticles to specific functional uptake pathways shared with EVs. Encouraging results in this regard have been obtained with EV-mimetic liposomes. For example, liposomes functionalized with integrin $\alpha 6\beta 4$ have been shown to be deliver miRNAs to recipient cells at higher efficiency than their non-functionalized counterparts [328]. Similarly, liposomes with a lipid composition resembling that of EVs have been demonstrated to exhibit improved uptake and cargo delivery capacity compared to simple PC/cholesterol liposomes [329]. As a last example, the "don't eat me signal" conveyed by EV-expressed CD47 has proven to be a rich source of inspiration for the design of synthetic nanoparticles with extended circulation times [330–332].

As an alternative to empowering synthetic drug delivery systems with EV components, identification of the functional components in EV preparations may allow enrichment of 'active' EV subpopulations for therapeutic applications. In addition, engineering strategies may be devised to selectively load therapeutic molecules in such subpopulations. However, a number of significant technical hurdles still have to be overcome for such strategies to be implemented in clinical practice (for reviews see [21,333]). A vital step in the development of EV-based therapeutics is the selection of an appropriate EV-producing cell source. As outlined in this review, EVs from different cell types (and different culturing conditions) can vastly differ in their composition and concomitant cell-specific interactions. As a striking example, recent work from our group showed that sgRNAs could not be functionally transferred to recipient HEK293T cells by co-culture with sgRNA-overexpressing HEK293T donor cells, whereas such transfer could be mediated by co-culture with, or isolated EVs from other cell sources such as tumor cells or MSCs [129]. Furthermore, most research on EVs is conducted on lab-scale, where EVs are typically isolated from relatively small volumes of liquids (<1 L) using technology that is not feasible for larger volumes (e.g. ultracentrifugation). In addition, such protocols generally yield EV preparations with variable degrees of purity and corresponding variable biological activity. For example, ultracentrifugation may result in co-isolation of contaminants and the formation of EV aggregates which show an altered biodistribution compared to EVs isolated using size exclusion chromatography [171]. In addition to the isolation process, the conditions under which EVs are best stored to preserve their functionality are still subject of debate and may need to be individually optimized per specific application [334]. Lastly, standardized 'potency assays' which can be used to perform quality control on EV preparations are still lacking. The development of such assays goes hand in hand with the identification of EV components and co-isolated contaminants which drive beneficial (or counteractive) effects.

Fortunately, these challenges are recognized by the EV research field and are continuously being addressed. For example, a recent study demonstrated the use of large-scale cell culture and EV isolation protocol to produce engineered EVs for various therapeutic applications [237]. Additionally, the EV research community is actively pursuing standardization and classification of EV preparations, for example through the publication of guidelines for EV characterization and functional assessment [97,335], which may aid in resolving reproducibility issues. Lastly, preclinical and clinical studies using EVs from various cellular sources are currently ongoing and have so far shown that (even allogeneic) EVs are well tolerated and that EV-based therapy is feasible [21]. Together, these efforts will pave the way for clinical translation of EV-based therapeutics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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