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Review

Bioengineering extracellular vesicle cargo for optimal therapeutic efficiency

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Extracellular vesicles (EVs) have the innate ability to carry proteins, lipids, and nucleic acids between cells, and thus these vesicles have gained much attention as potential therapeutic delivery vehicles. Many strategies have been explored to enhance the loading of specific cargoes of interest into EVs, which could result in the delivery of more therapeutic to recipient cells, thus enhancing therapeutic efficacy. In this review, we discuss the natural biogenesis of EVs, the mechanism by which proteins and nucleic acids are selected for inclusion in EVs, and novel methods that have been employed to enhance loading of specific cargoes into EVs. As well, we discuss biodistribution of administered EVs *in vivo* and summarize clinical trials that have attempted to harness the therapeutic potential of EVs.

INTRODUCTION

Extracellular vesicles (EVs) are small membrane-bound particles secreted from cells into the extracellular space.[1] Initially, EVs were thought be involved only in the elimination of cellular waste, but EVs actually perform an array of physiological functions in the body.[2,3] These vesicles shuttle proteins, lipids, and nucleic acids between cells to mediate intercellular communication.[4] The specific function and physical composition of EVs is determined by both the type and physiological state of the cell from which the EVs are released.[1]

EVs are a heterogeneous population of particles, of which there are three main subtypes: exosomes, microvesicles (MVs), and apoptotic bodies. Exosomes have a diameter of between approximately 40 and 150 nm and are the smallest of the three subtypes.[5,6] Exosomes are formed through invagination of the membrane of multivesicular bodies (MVBs) and are subsequently released into the extracellular environment following fusion of the MVB with the plasma membrane of the cell.[3] In contrast, MVs, which are approximately 40–1000 nm in diameter, are generated by budding at the plasma membrane.[1,6,7] The largest subtype of EVs are apoptotic bodies, >1,000-nm-diameter particles generated by dying cells through blebbing from the plasma membrane.[6] Many other subtypes of EVs have been described, such as large oncosomes and arrestin domain-containing protein 1 (ARRDC1)-mediated MVs (ARMMs),[7] but these particles are less-well characterized than the three main EVs. Differentiating between the various subtypes of EVs can be challenging, as the subtypes frequently have common cargo content and overlapping particle diameter.[8] In the absence of actually observing the biogenesis of the vesicle (i.e., whether the particle arises from fusion of the MVB with the plasma membrane or directly from budding at the plasma membrane), it is more accurate in an experimental context to refer to EVs as either small ($\leq$200 nm) or large (>200 nm) EVs. This review uses the term that was used by the original publication, whether that is "EV," "exosomes," or "MVs."

EVs have many attractive features as therapeutics for the treatment of disease. The innate ability of EVs to shuttle cargo between cells makes the vesicles attractive therapeutic vehicles. Some EVs can target their uptake to specific cell types based on proteins presented on the surface of the EV and a complementary "receptor" on the surface of the target cell.[9] Many studies have shown EVs administered to an allogeneic recipient induce minimal immunogenicity *in vitro* or *in vivo*.[10–12] Some populations of EVs even have transmembrane proteins, such as CD24,[13,14] CD47,[15] and programmed death-ligand 1 (PD-L1),[16,17] that allow the EVs to avoid phagocytosis by macrophages, thus providing the EVs with greater opportunity to deliver cargo to the desired target cell. Given these distinct advantages, it is unsurprising that many groups are exploring the use of EVs as therapeutic delivery vehicles and also developing methods to target specific proteins or nucleic acids into the EVs.

NATURAL BIOLOGY OF EVs

Biogenesis of exosomes and MVs

Exosomes and MVs are generated through different mechanisms of biogenesis, which each involve several sequential steps (Figure 1). For exosomes, the process begins with invagination of the plasma membrane, resulting in the formation of early endosomes inside the cell. The early endosome matures into the late endosome in a process mediated by Ras-associated binding (Rab) GTPases, notably those involved in the conversion of $Rab5^+$ endosomes to $Rab7^+$ endosomes.[18,19] This endosome maturation step is followed by formation of intraluminal vesicles (ILVs) within the MVB through invagination

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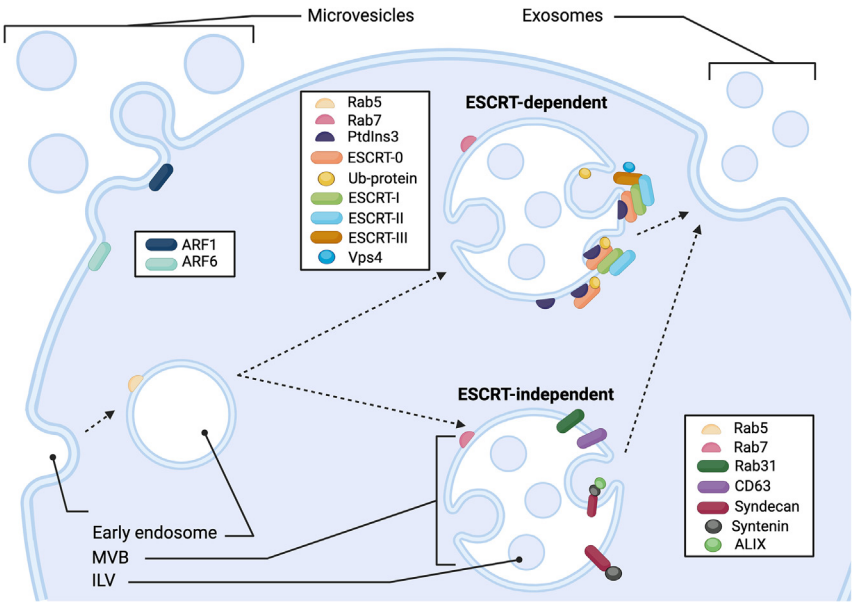

Figure 1. Biogenesis of exosomes and microvesicles
Invagination of the plasma membrane of the cell leads to development of the early endosome. The early endosome matures into the MVB, at which point Rab5 is replaced by Rab7. In ESCRT-dependent exosome biogenesis, ESCRT-0 interacts with Ub-proteins and binds PtdIns3P on the membrane of the endosome. ESCRT-I and ESCRT-II are then recruited, driving the invagination of the endosomal membrane. ESCRT-III completes ILV formation and is dissociated by Vps4. In ESCRT-independent exosome biogenesis, ILV formation can be driven by Rab31, CD63, or through the syndecan-syntenin-ALIX pathway. In both types of biogenesis, the MVB fuses with the cell membrane and leads to the release of ILV as exosomes. In microvesicle biogenesis, outward budding of the cell membrane and pinching off of the membrane results in the release of microvesicles. This process involves ARF1 and ARF6. ESCRT, endosomal sorting complex required for transport; ILV, intraluminal vesicle; MVB, multivesicular body; PtdIns3, phosphoinositide; Rab, ras-associated GTPases; Ub, ubiquinated.

of the endosomal membrane, mediated by the endosomal sorting complex required for transport (ESCRT) machinery, composed of the ESCRT-0, -I, -II, and -III multiprotein complexes.[20] Hepatocyte growth factor-regulated tyrosine kinase (HRS), a subunit of the ESCRT-0 complex, is recruited to the endosomal membrane where it binds to phosphoinositide (PtdIns3P) on the endosome membrane, initiating the process of ILV formation.[21–24] The tumor susceptibility gene 101 (TSG101) subunit of the ESCRT-I complex binds to HRS and, with ESCRT-II, drives the invagination of the endosomal membrane.[25,26] ILV formation within the MVB is completed through membrane scission mediated by ESCRT-III, which is composed of vacuolar protein-sorting-associated protein 20 (Vps20), sucrose non-fermenting protein 7 (Snf7), Vsp24, and Vps2.[27,28] The AAA ATPase VPS4 disassembles the ESCRT-III complex, disassociating its subunits from the MVB and enabling their reuse.[29,30]

Exosome biogenesis can still occur in the absence of the ESCRT machinery,[31] and several cellular proteins have been implicated in mediating this ESCRT-independent pathway. Rab31 can be recruited to the endosome, where it is phosphorylated by epidermal growth factor receptor (EGFR) present on the surface of the endosome.[32] Rab31 then interacts with flotillin proteins, thus driving ILV formation. Similarly, tetraspanins, such as CD9, CD81, and CD63, are thought to be involved in ESCRT-independent mechanisms of exosome biogenesis.[33–35] Indeed, depletion of CD9 leads to a reduction in formation of early endosomes and decreased exosome secretion.[36] Similar to HRS, CD63 can engage the endosomal membrane, permitting ILV formation even in the absence of ESCRT-0.[37] The syndecan-syntenin-ALG-associated protein X (ALIX) pathway for exosome biogenesis also does not require ESCRT-0; syndecan in the membrane of the MVB interacts with syntenin, which promotes ILV biogenesis through interaction with ALIX.[38,39]

In both ESCRT-dependent and -independent exosome biogenesis, the MVB ultimately fuses with the plasma membrane of the cell to release ILVs into the extracellular space as exosomes.[40,41] A portion of the exosomes released from the ILVs may remain associated with the plasma membrane of the cell, mediated by tetherin located on the surface of both the cell and exosome.[42] Alternatively, the MVB may fuse with a lysosome, leading to degradation of the MVB contents due to the action of hydrolases and proteases contained in the fused vesicle.[43]

MVs differ from exosomes in that they originate from the outward budding and fission of the cell plasma membrane, but much less is known about their mechanism of biogenesis.[44] The outward budding of the plasma membrane is accompanied by distinct changes in protein and lipid content of the membrane that leads to a change in the curvature and rigidity of the membrane. ADP-ribosylation factor 1 (ARF1) can use myosin light-chain kinase (MLCK) to activate contractile machinery to allow for pinching off of the plasma membrane to release MVs into the extracellular environment.[45] ARF6 can also activate the contractile machinery via MLCK, and can regulate the selective recruitment of some proteins into MVs.[46] ARRDC1 can interact with TSG101 to release a subpopulation of MVs called ARMMs.[7] ARRDC1 is localized to the plasma membrane, and recruits TSG101 to the membrane from the endosome. ARMMs are released into the extracellular space following ubiquitination of ARRDC1.[7]

Cargo loading

Thousands of proteins have been identified as EV cargo, and these proteins are cataloged in databases such as Vesiclepedia (http://www.microvesicles.org). Some of these proteins are actively loaded into EVs, such as through the processes detailed below, but many may be passively loaded, as any protein or nucleic acid that is in

the general vicinity of EV biogenesis may ultimately be incorporated into the EVs.

Many proteins involved in the biogenesis of EVs are retained within mature EVs. These same proteins also frequently mediate the selective loading of other cellular proteins, which are recruited to EVs as a consequence of natural protein-protein interactions. As mentioned, one of the possible fates of the MVB is to fuse with the lysosome, leading to degradation of the MVB contents.[47] Indeed, some of the protein contents of the ILV are ubiquitinated—a cellular protein modification commonly used to tag a protein for degradation and disposal.[48,49] The ESCRT machinery, along with a number of accessory proteins, plays a role in biomolecule sorting of exosomal cargo and can specifically interact with ubiquitinated proteins to facilitate their loading into EVs.[50] The HRS and signal transducing adaptor molecule (STAM) subunits of the ESCRT-0 complex both contain ubiquitin identification motifs, and can recruit ubiquitinated protein cargo to the endosomal membrane, leading to their incorporation into exosomes.[51] Similarly, the TSG101 and ubiquitin-associated protein 1 (UBAP1) subunits of ESCRT-I each contain a ubiquitin binding domain that can also recruit ubiquitinated cargo to the endosomal membrane.[52,53] The Vps45 domain of ESCRT-II also can bind ubiquitinated proteins and plays a role in loading of ubiquitinated cargo.[54] EVs are enriched in many tetraspanins, including CD9, CD37, CD63, CD81, and CD82.[55,56] CD9 and CD63 can facilitate the incorporation of major histocompatibility complex (MHC) class II proteins into exosomes.[57,58]

Proteins may be selectively included into MVs during biogenesis, such as proteins transported via the ARF6-regulated endosomal recycling pathway, including β1 integrin receptors and vesicle-associated membrane protein 3 (VAMP3).[59,60] RAB22A, which is overexpressed in many breast cancer cells and can selectively recruit proteins to MVs, facilitates an increased production of MVs under hypoxic conditions.[61] These hypoxia-induced MVs were found to stimulate focal adhesion formation, extracellular matrix deposition, and enhance lung colonization by breast cancer cells in an RAB22A-dependent manner *in vitro*.

Ribonucleic acid (RNA)-binding proteins (RBPs) make up approximately 25% of the total proteins contained within EVs, and thus RNAs are also common cargo within EVs.[62] There is a lack of consensus on whether full-length messenger RNA (mRNA) can be incorporated into EVs, as evidence suggests that fragmented transcripts,[63,64] full-length transcripts,[65,66] or both forms[67–69] are loaded into EVs. Many proteins are thought to mediate the loading of microRNA (miRNA) into EVs, including various heterogeneous nuclear ribonucleoproteins (hnRNPs),[70–72] major vault protein (MVP),[73] and Y-box-binding protein-1 (YBX-1).[74,75] Several sequence motifs in RNA have been identified that are thought to lead to the enrichment of specific RNAs within EVs.[76–78] One such motif is CGGGAG that can be recognized by two RBPs, fused in sarcoma (Fus) and Alyref, promoting the loading of miRNA into EVs.[77] Exploitation of these natural pathways for cargo loading can be used to selectively enhance uptake of RNA into EVs, as discussed below.

Table 1. Engineered cargo loading strategies

Cargo	Strategy	Reference
Protein	Overexpression of protein	Nash et al.[83]
	Fusion proteins	
	CD63	McCann et al.[88]
	LAMP2B	Alvarez-Erviti et al.[89]
	GPI	Kooijmans et al.[90]
	PDGFR	Ohno et al.[91]
	Lactadherin	Rountree et al.[92]
	PTGFRN	Dooley et al.[93]
	BASP1	Dooley et al.[93]
	Nef	Manfredi et al.[94]
	Protein:protein association	
	KFERQ motif	Ferreira et al.[95]
	EXPLORs system	Yim et al.[96]
	Rapamycin-FKBP-FRB system	Somiya and Kuroda[97]
	WW-domain	Sterzenbach et al.[98]
	Ub tag	Cheng and Schorey[99]
RNA	EXOmotifs	Villarroya-Beltri et al.[70]
	Incorporation of pre-miR-451 backbone	Reshke et al.[100]
	GUGCC-containing motif	Bolukbasi et al.[76]
	TAMEL system	Hung and Leonard[101]
	G58T peptide	Dar et al.[102]

ENGINEERED EV CARGO LOADING STRATEGIES

EVs can be loaded with cargo of interest using either exogenous[79–81] or endogenous[82,83] loading. In exogenous loading, EVs are first harvested from a biofluid, and then cargo is loaded. Many different methods have been used to achieve exogenous loading, including electroporation, sonication, freeze-thaw cycles, extrusion, and the use of pore-forming agents.[81,84–86] Unfortunately, these approaches can result in permanent damage of the EV membrane, as many of these methods rely on transient disruption of the membrane to load the cargo. The efficiency of electroporation was found to be extremely low, with less than 0.05% encapsulation efficiency.[87] In endogenous loading, EVs are isolated from cells that have been manipulated in some way, for example through engineering to overexpress a particular therapeutic protein, so that the protein is incorporated into the EVs during their natural formation.[82,83] Several strategies have been developed to increase the efficiency of endogenous cargo loading, thus increasing the concentration of the cargo of interest within EVs (Table 1).

Protein

EV cargo content usually reflects the state of the cell from which they originate, and simply overexpressing a protein of interest within donor cells can lead to release of EVs loaded with high levels of that protein.[103,104] For example, we showed that plasmid- or adenovirus-mediated overexpression of survival motor neuron (SMN)

protein in donor cells led to release of exosomes with elevated concentrations of SMN protein.[83] Countless studies have shown a similar effect with numerous different proteins.

Fusion proteins

A popular approach to enhance uptake of a desired protein into EVs is to create a fusion protein with a second protein that is naturally incorporated into EVs. Fusion of protein cargo to CD63 can increase the presentation of that cargo on the surface of the EVs. CD63 is a tetraspanin found on the surface of cells, and is consequently also found in endosomes and on the surface of EVs.[55] HEK293T cells transfected with a plasmid encoding a CD63-emerald green fluorescent protein (emGFP) fusion protein incorporated more emGFP into EVs compared with cells transfected with plasmid expressing emGFP alone.[88] GFP has been conjugated to several other EV proteins, and vesicle association of GFP was more efficient when coupled to membrane-associated proteins, such as CD9, CD63, and CD81, compared with proteins normally found in the cytosol of EVs, such as ALIX, Flotillin-2, or native-uncoupled GFP.[105,106] Conjugation of ovalbumin (OVA) antigen (Ag) to CD63 (CD63-OVA) dramatically increased the presence of OVA on the EV surface.[107] Moreover, CD63-OVA EVs elicited a stronger Ag-specific immune response than OVA EVs or OVA alone following immunization of mice. Fusion of the neuron-specific rabies viral glycoprotein (RVG) peptide to the EV membrane protein lysosome-associated membrane glycoprotein 2B (LAMP2B) resulted in presentation of RVG peptide on the surface of exosomes, which was used to enhance targeting of the vesicles to neurons *in vivo*.[89,108] Glycosylphosphatidylinositol (GPI),[90] platelet-derived growth factor receptors (PDGFRs),[91] and lactadherin (C1C2 domain),[92] all of which are naturally found in the membrane of EVs, have also been used to display proteins of interest on the surface of EVs.

Proteins prostaglandin F2 receptor inhibitor (PTGFRN), a member of the immunoglobulin superfamily, and brain acid soluble protein 1 (BASP1), a member of the myristoylated alanine-rich C-kinase substrate protein family, are enriched in EVs compared with non-EV fractions of conditioned medium as assessed by liquid chromatography mass spectrometry.[93] Cargo can be loaded into EVs with high efficiency when fused to the PTGFRN or BASP1. Fusion of GFP to the intracellular domains of PTGFRN or BASP1 resulted in enhanced loading of GFP into EVs compared with GFP alone.[93] These same fusion proteins led to higher levels of association of GFP with EVs than similar fusion proteins with LAMP2B, CD9, CD63, or CD81, which may simply reflect the relative abundance of the various membrane proteins naturally found in EVs. As PTGFRN is a single-pass transmembrane protein, target proteins can be fused to the C or N terminus, resulting in the presence of the target protein on either the extra- or intravesicular side of the EV membrane, respectively. Fusion to PTGFRN permitted display of several target molecules on the surface of EVs, including cytokines and antigens.[93] Fusion to BASP1 increased loading of several proteins into the lumen of EVs, including clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) protein.[93]

The human immunodeficiency virus (HIV)-1 negative regulatory factor (Nef) protein is naturally incorporated into exosomes at high levels.[94] Nef promotes viral replication of HIV-1 by enabling immune escape of infected cells.[109,110] Use of an engineered mutant of Nef (Nef^{mut}), deficient in most functions of intact Nef protein, was able to efficiently load several fusion proteins into exosomes, including GFP and Ag from several viruses.[94,111] These Ag-loaded exosomes effectively primed cytotoxic T lymphocyte immunity in mice.[111]

Protein:protein association

Cargo loading can also be increased through use of peptide motifs that mediate non-covalent interaction with proteins normally recruited into EVs. Proteomic and bioinformatic analysis of EVs derived from cells either expressing or lacking LAMP2A, an EV scaffolding protein, showed that proteins containing a KFERQ motif were under-represented in the LAMP2A-deficient EVs.[95] Fusion of the KFERQ motif to mCherry led to binding of mCherry to LAMP2A, as assessed by co-immunoprecipitation, and enhanced uptake of the protein into EVs compared with mCherry alone. The authors also showed that proteins tagged with KFERQ motifs were loaded into EVs independent of the ESCRT machinery.

An elegant exosomal loading technique was developed called exosomes for protein loading via optically reversible protein-protein interactions (EXPLORs).[96] This system is composed of two components. First, photoreceptor cryptochrome 2 (CRY2) protein was fused to target cargo, such as mCherry. Second, truncated CRY-interacting basic-helix-loop-helix 1 (CIBN) protein was fused to the intraluminal side of CD9, which resulted in presentation of CIBN on the external surface of the MVB. Application of blue light induced association of CIB1 and CRY2, recruiting the mCherry-CRY2 to the membrane of the MVB to transiently interact with CIBN-CD9, thus drawing the protein into the exosome during ILV formation. Once the blue light was turned off, mCherry-CRY2 disassociated from CIBN-CD9, resulting in release of the protein into the intraluminal space of the exosomes, ensuring that the target protein was not irreversibly stuck to the exosome membrane, and ultimately allowing efficient delivery of the target protein to recipient cells. This study further demonstrated functional delivery of various proteins, such as Bax-mCherry:EXPLORs to HeLa cells, which caused a release of cytochrome *c* from the mitochondria, leading to caspase-dependent apoptosis. Delivery of srIκB-mCherry:EXPLORs (super-repressor IκB [srIκB], an inhibitor of nuclear factor [NF]-κB) to HeLa cells reduced tumor necrosis factor-α (TNF-α)-induced translocation of NF-κB to the nucleus of the cells, thereby decreasing the expression of genes related to survival and inflammation of cancerous cells.[112] Yim et al.[96] used EXPLORs to deliver Cre protein to a transgenic enhanced yellow fluorescent protein (EYFP) reporter mouse. Ventrolateral injection of the brain with Cre:EXPLORs led to EYFP protein expression mainly in neurons in the ventrolateral part of the brain of these mice as assessed 96 h post-injection.

Recently, a system similar to EXPLORs was developed that used rapamycin to induce interaction of specific fusion proteins to enhance their incorporation into EVs.[97] Rapamycin naturally induces

heterodimerization of FK506 binding protein (FKBP) and FKBO12-rapamycin-binding (FRB) domains.[113] CD81 (naturally found in the membrane of exosomes) was fused to FKBP, and FRB was fused to the tetracycline transactivator (tTa) protein. HEK293T cells were transfected with pCD81-FKBP, pFRB-tTa, and pVSV-G, a vesicular stomatitis virus fusogenic glycoprotein known to enhance delivery efficiency of EVs,[114,115] and the cells were cultured in the presence or absence of rapamycin. The FRB-tTa fusion protein was found in much higher quantity in EVs isolated from cells cultured in rapamycin, and these EVs delivered more tTa to recipient cells, compared with EVs isolated from cells not treated with rapamycin, as measured by induction of expression from a tTa-controlled reporter gene present in the target cells. Cytoplasmic delivery of an FRB-fused protein was also demonstrated using a split NanoLuc luciferase reporter, in which one part of NanoLuc was fused to FRB and the complementing fragment was expressed in the recipient cells, thus emitting luminescence only if the FRB-fused protein was successfully internalized and was available to complement NanoLuc activity. In place of CD81, other EV-enriched proteins were tested using the EXPLORs approach. CD63-FKBP and HIV Gag-FKBP showed weaker reporter gene expression in recipient cells than CD81-FKBP as detected by the split NanoLuc reporter, but were still significantly greater than control. The rapamycin-FKBP-FRB EV cargo loading method was also used to package Cas9 protein and single guide RNA (sgRNA) into EVs to induce therapeutic exon skipping in reporter mice and tissue culture, including in Duchenne's muscular dystrophy (DMD) patient-induced pluripotent stem cells (iPSCs).[116] In that study, exon 45 of dystrophin was removed using this method to restore the reading frame and dystrophin protein expression in the DMD patient-derived iPSCs containing exon 44 or 46–47 deletions. A VGV-G-free method of rapamycin-FKBP-FRB EV cargo loading has been developed through the use of Syncytin-1, an endogenous human fusogen protein.[117]

A ubiquitin-based technique has also been used to incorporate target proteins into exosomes.[98] Fusion of a WW-domain (a 35-amino acid motif containing two conserved tryptophans) onto a protein allows the protein to interact with Nedd4 family interacting protein 1 (Ndfip1), which facilitates Nedd4-mediated ubiquitination of the target protein, and subsequent preferential loading into EVs.[98,118,119] For example, transient expression of Cre protein alone within the producer cells did not result in appreciable loading of the Cre protein into exosomes, whereas WW-Cre protein was loaded into exosomes, but only if Ndfip1 was expressed in the producer HEK293 cells.[98] The requirement for Ndfip1 was further demonstrated through the use of murine embryonic fibroblasts (MEFs) isolated from Ndfip1 knockout (KO) mice; WW-Cre was not effectively loaded into exosomes in the Ndfip1-KO MEFs. Intranasal delivery of WW-Cre exosomes in transgenic reporter mice that can inducibly express tdTomato led to Cre-mediated recombination in the brain.

Another method that took advantage of the natural ubiquitin-based protein sorting system involved use of an engineered tag that mimics ubiquitin.[99] This method used a modified ubiquitin that was deleted of the two C-terminal glycine residues of ubiquitin (designated cUb), which improves the half-life of the protein.[120] Expression of a fusion protein composed of cUb and the *Mycobacterium tuberculosis* proteins Ag85B and early secreted antigen target 6 kDa (ESAT6) led to a 10-fold increase in loading of the fusion protein into exosomes compared with protein without the cUb tag.[99] Exosomes containing the Ag85B-ESAT6-cUb fusion protein were able to induce a stronger antigen-specific immune response in naive mice compared with exosomes isolated from cells expressing Ag85B-ESAT6 lacking the cUb tag. One caveat of this study is that it was not determined whether the cUb tag increases the immunogenicity of the fusion protein.

RNA

As mentioned, both deoxyribonucleic acid (DNA) and RNA, including miRNA, are found naturally in EVs. Numerous studies have analyzed the miRNA composition of EVs isolated from specific cell types or pathological conditions, such as cancer,[121,122] to gain insight into the disease state or identify potential new biomarkers. miRNAs released in cancer cell-derived EVs can have profound effects on neighboring cells, including promoting angiogenesis and tumorigenesis.[123,124] However, quantitative studies have shown that miRNAs are only present at very low levels in EVs, ranging from one copy of a given miRNA per EV to one copy per 10^6 EV,[125] and thus techniques must be developed to increase the efficiency of miRNA loading. Several proteins have been implicated in facilitating or chaperoning the loading of miRNAs into EVs. Sumoylated heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) binds a subset of miRNA through recognition of specific sequence motifs, called EXOmotifs, mediating their loading into exosomes.[70] Annexin A2 (AXA2) has also been shown to play a role in loading of miRNA into EVs.[126] Small interfering RNA (siRNA) silencing of AXA2 significantly decreased the amount of miRNA loaded into EVs in tissue culture.

Bioinformatic analysis of all miRNAs found in EVs isolated from MEFs, MDA-MB-231 human breast cancer cells, and NSC-34 motor neuron-like cells showed that pre-miR-451 is selectively enriched within EVs, suggesting it may contain an RNA motif that mediates preferential loading.[100] Indeed, incorporation of other miRNA sequences into the pre-miR-451 hairpin enhanced their loading into EVs.[100,127] Incorporation of siRNAs targeting GFP, tetracycline repressor, or superoxide dismutase 1 (SOD1) into the pre-miR-451 backbone caused these siRNAs to be enriched in EVs.[100] Incorporation in the pre-miR-451 backbone of siRNA targeting GFP led to an ~3,700-fold increase in incorporation of the modified siRNA into EVs and was very effective in silencing GFP expression in primary motor neurons isolated from GFP transgenic mice. When these EVs were administered to GFP transgenic mice by tail vein injection, ~50% GFP knockdown was observed in the small intestine and liver of the animals, while no knockdown was noted in the heart, lungs, or brown fat. In the G93A SOD1 mouse model of amyotrophic lateral sclerosis, systemic administration of EVs loaded with siRNA targeting SOD1 in the pre-miR-451 backbone resulted in knockdown of SOD1 by ~60% in the liver and ~75% in the small intestine. This study clearly shows the promise of this approach in improving

incorporation of a desired siRNA into EVs, but also highlights the challenge of uneven distribution of EVs following systemic delivery.

Glyceraldehyde-3-dehydrogenase (GAPDH) can associate with the surface of EVs through a phosphatidylserine-binding domain, termed the G58 peptide.[102] G58T, a fusion protein consisting of the G58 peptide and the RNA-binding domain of TAR RNA-binding protein 2 (TARBP2), was able to load siRNA onto EVs with high efficiency without the need to incorporate a targeting sequence into the siRNA. Delivery of EVs loaded with siRNA targeting GAPDH was able to reduce the levels of the protein to ∼15% of untreated N2a recipient cells. These researchers also utilized the G58T system to develop EVs that contained a mixture of siRNA directed toward Huntingtin protein (Htt), designated G58TF/siRNA-RVG-EVs. The EVs also had an RVG peptide on the surface to target EVs to neurons. The G58T chimeric protein was further engineered to include a flock house nodovirus (FHV) domain,[102] which has been shown to enhance release of cargo from late endosomes.[128] In the Q140 mouse model of Huntington's disease, administration of G58TF/siRNA-RVG-EVs once a week for 4 weeks resulted in ∼40% reduction in Htt expression in the mouse brain cortex.[102]

For mRNAs, a 25-nucleotide sequence in the 3′ untranslated region (UTR) of some mRNA appears to be involved in EV loading.[76] This sequence binds miR-1289 and includes a GUGCC core presented on a stem-loop structure. Incorporation of this sequence into the 3′ UTR of a reporter mRNA led to increased loading into MVs, although the protein or proteins responsible for the increased loading of GUGCC motif-containing mRNA remains unknown.

A novel technique for loading RNA into EVs has been developed called Targeted and Modular EV Loading (TAMEL).[101] The coat protein from bacteriophage MS2 naturally binds to a stem-loop structure present in the viral RNA genome.[129] For the TAMEL system, the MS2 coat protein was fused to LAMP2B, a protein naturally recruited to EVs, and the stem-loop structure from the MS2 genome was engineered into the cargo RNA.[101] The TAMEL approach was able to enhance the loading of cargo RNA into EVs up to 6-fold. When the TAMEL approach was used in cells also expressing VSV-G, a 40-fold increase in cargo RNA loading into EVs was observed. The authors hypothesized that this increase in loading may be due to the ability of VSV-G to augment uptake of cargo into EVs via binding to the low-density lipoprotein (LDL) receptor, which normally also plays a role in VGV-G-mediated lentiviral delivery.[130,131]

Taken together, these studies show that there are many elegant techniques that can be exploited to load a specific nucleic acid or protein into EVs. However, once a product is developed, the next hurdle is producing and purifying sufficient product to perform *in vitro* and/or *in vivo* characterization of efficacy.

ISOLATION AND PURIFICATION OF EVs

There are several methods currently in use for isolation of EVs. The most common methods include differential ultracentrifugation, size-exclusion chromatography, filter concentration, polyethylene glycol (PEG) precipitation, density gradients, and tangential flow filtration (TFF).[8] Each method has a different degree of specificity and recovery, and unique advantages and disadvantages. Many insightful reviews have discussed these different isolation methods in depth, as well as challenges associated with large-scale manufacturing of therapeutic EVs.[132–136] The method of EV isolation employed for a particular study in part depends on the intended application. For example, while PEG-based methods may be ideal for isolation of EVs from small volumes of biological fluids for biomarker studies, TFF may be more appropriate for isolation of EVs from large volumes of tissue culture medium for use in human clinical trials. Regardless of the method used to isolate the EVs, the resulting product must be fully characterized to determine the purity, quality and composition (e.g., particle size distribution, concentration) of the sample. The International Society for Extracellular Vesicles has released a series of consensus papers entitled "Minimal information for studies of extracellular vesicles (MISEV)," most recently in 2023 (MISEV2023),[8] that outline preferred methods to evaluate EV samples. If adopted throughout the field, the MISEV guidelines should lead to greater clarity and reproducibility of all EV studies.

BIODISTRIBUTION OF EVs

EVs are released by virtually every cell type and found in all bodily fluids, including blood,[137] urine,[138] saliva,[139] and cerebrospinal fluid (CSF).[140] EVs from different cell types will have specificity for different target tissues, based on a combination of the composition of proteins present on the EV surface and complementary receptors found on the recipient cell.[141,142] This is also true for EVs mass-produced in tissue culture for *in vivo* studies. However, the method of EV administration will also influence where delivered EVs accumulate.[9]

Intravenous administration

Many groups have demonstrated that EVs isolated from a variety of cell types accumulate in liver, spleen, kidney, and lungs following tail vein injection in mice, although the primary tissue in which the EVs accumulated varied between studies.[143–146] In one study, intravenous (i.v.) administration of EVs resulted in preferential uptake by macrophages in the liver and spleen and endothelial cells in the lungs in mice.[147] In another study, EVs isolated from mesenchymal stem cells (MSCs) labeled with fluorescent dye were found in not only the liver and spleen, but also in the femur, tibia, and spinal cord following i.v. administration in a radiation injury bone marrow mouse model.[148] Accumulation of MSC-derived EVs at the site of injured bone marrow could be detected as soon as 1 h post-injection (hpi) and fluorescence was statistically unchanged between 2 and 24 hpi. The authors interpreted this as maintenance in concentration of therapeutic EVs at the target tissue over time. Many of the studies listed below compared i.v. administration with intraperitoneal (i.p.) or intranasal administration, and further demonstrate the wide variety of tissues EVs distribute to following i.v. administration.

Intraperitoneal administration

The i.p. administration of therapeutic EVs has also been investigated in mouse models. MSC-derived EVs were administered i.p. in healthy

and pancreatic tumor-bearing mice.[149] In both treatment groups at 6 hpi, EVs accumulated in the pancreas, but showed especially high accumulation within the tumors in the pancreas. The study reported that accumulation of EVs in the pancreas was greater than EV accumulation within the liver, spleen, or lungs in both tumor-bearing mice and controls. EVs were also present within pancreatic tumors following i.v. administration of EVs, although to a lesser extent than EVs administered i.p., and greater signal intensity was found in the liver, lungs, and spleen compared with EVs administered i.p.. Accumulation of signal intensity in the pancreatic tumors at 24 and 48 hpi suggested tumor-specific EV retention. In another study that compared methods of administration, EVs isolated from HEK293T cells were labeled with the fluorescent lipophilic tracer DiR and administered i.p., i.v., or subcutaneously in mice.[9] All treatment groups had significant accumulation of EVs in the liver, but i.v. administration led to the highest level of liver uptake of the three delivery routes examined. The i.v. administration group also had the greatest accumulation of EVs in the spleen. Accumulation of EVs in the lungs was similar in mice treated subcutaneously or i.v., but lower in mice treated i.p. In contrast, EV accumulation in the pancreas was greatest in mice treated i.p. and lowest in mice treated i.v. Significant accumulation of EVs was noted in the gastrointestinal tract of mice treated i.p. and subcutaneously, and to a lesser extent in mice treated i.v. Low levels of EV accumulation were also noted in brain, heart, kidney, and quadriceps muscle of all treated mice.

Intranasal administration

Although EVs are naturally capable of transiting the blood-brain barrier,[150,151] intranasal delivery of EVs has been used in an attempt to increase delivery of EVs to the brain. Following intranasal delivery, particles, such as EVs, are thought to be transported to the brain via transcytosis of olfactory receptor neurons, which connects the nasal cavity and olfactory bulb.[152] In a study comparing methods of administration, EVs isolated from MSC were administered intranasally or i.v. to mice, although mice treated i.v. were administered a four times higher dose of EVs than the intranasal group.[153] Twenty-four hours after administration, the greatest accumulation of EVs was in the liver, lungs, and spleen of i.v.-treated mice, with some accumulation in the heart, kidney, and brain. In contrast, relatively high accumulation of EVs was noted in the brain of mice treated intranasally, with relatively low levels in the heart and gut. In a similar study, increasing doses of EVs containing luciferase were administered to non-human primates (NHPs) either i.v. or intranasally.[154] Intranasal administration at all doses resulted in little-to-no detectable luminescence in plasma at a variety of time points spanning from 1 min to 24 h post-administration. In contrast, doses administered i.v. produced a luminescent signal in plasma but, surprisingly, higher doses of EVs were cleared more rapidly from plasma than lower doses, for reasons that are unclear. In CSF, luminescence was detected with high doses of EVs delivered i.v., whereas no luminescence was detected in mice treated intranasally. Also in this study, NHPs were administered a fixed-dose of EVs either intranasally or i.v., and tissues were collected 1 hpi. Greater luminescence was noted in liver, spleen, lung, and colon tissue of i.v.-treated NHPs compared with the intranasal administration group, whereas similar luminescence was noted in kidney and heart tissue of both administration groups. In general, similar luminescence was detected in the brain of intranasally and i.v. treated NHPs; however, greater luminescence was noted in the cerebellum of i.v.-treated NHPs, and in the midbrain of intranasally treated NHPs. These researchers also examined biodistribution of EVs in mice, and observed enhanced uptake of EVs in the liver, spleen, kidney, brain, and colon of i.v.-treated mice compared with mice treated intranasally. Thus, the hypothesis that intranasal delivery of EVs may lead to enhanced delivery to the brain may not be supported by the experimental data. Overall, these studies highlight the impact of method of administration on biodistribution of therapeutic EVs.

EV-BASED THERAPEUTICS IN HUMANS

Although this review has focused exclusively on EVs derived from mammalian systems, the first significant use of EV-like particles in humans was the development and application of anti-meningitis type B vaccines derived from bacterial outer membrane vesicles (OMVs).[155] These OMVs are isolated from *Neisseria meningitis* type B bacteria, and are naturally released through blebbing of the outer membrane.[156,157] The OMVs contain many bacterial proteins and lipids, and can stimulate anti-bacterial immunity when administered as a vaccine in humans.[158,159] One such vaccine, VA-MENGOC-BC15, has been administered in over 60 million doses in Latin America since it was first developed in 1989, and has proven both safe and effective.[155,156] EVs derived from mammalian sources have not yet been approved for any human disease indication, but have been studied in several clinical trials, as outlined below.

Cancer therapy

EVs have gained significant attention as potential therapeutic vehicles to efficiently deliver chemotherapeutic agents to cancer cells. MV isolated from tumor cells *in vitro* have the capacity to effectively deliver chemotherapeutic agents to the tumor microenvironment *in vivo*.[160–162] One clinical trial examined the ability of MVs containing chemotherapeutic drugs to reverse drug resistance of tumor-repopulating cells (TRCs).[161] Six end-stage lung cancer patients with metastatic malignant pleural effusion were included in the study. Primary tumor cells collected from malignant fluids prior to MV treatment showed resistance to the chemotherapeutic drug cisplatin (Cis) due in part to upregulated expression of multidrug-resistance genes. In this study, A549 cells, a lung carcinoma cell line, were co-cultured with Cis, during which Cis was taken up by these cells, thus releasing MVs loaded with Cis, which were subsequently isolated by centrifugation. Three patients were treated with these Cis-loaded MVs (MV-Cis), administered by intrathoracic injection, while the remaining three patients were treated with intrapleural Cis injections. Both treatment groups received daily injections; however, the dose of MVs administered to the patients was not indicated by the authors. After 7 days of treatment, the MV-Cis treatment group had a >95% decrease in tumor cells in pleural fluid, whereas patients treated with Cis alone did not show a significant reduction in tumor cells. These data indicate encapsulation of Cis within MVs may reverse TRC drug resistance.

In another similar clinical trial, patients were treated with A549 cell-derived MVs loaded with the chemotherapeutic drug methotrexate (MTX) (MV-MTX).[162] Eleven patients with advanced lung cancer and malignant pleural effusion were included in this trial. MV-MTX was administered by intrapleural infusion every other day for 12 days, totaling six infusions. Response to treatment was determined by objective clinical responses as per the British Thoracic Society Pleural Effusion guidelines.[163] Decreased pleural effusion volume was observed in 10 of 11 patients (91%) at 28 days post-treatment.[162] Four of 11 patients (36%) showed a complete response, six of 11 (54%) showed a partial response, and one of 11 (9%) showed no response. Analysis of pleural fluid collected pre- and post-treatment from eight patients showed a reduction in tumor cells.

Another study examined the safety and efficacy of MV-MTX combined with the chemotherapeutics pemetrexed and Cis for treatment of malignant pleural effusion in advanced non-squamous non-small cell lung cancer (NSCLC).[164] A total of 86 chemotherapy-naive patients with non-squamous NSCLC and malignant pleural effusion were enrolled in this study. All 86 patients received pemetrexed and Cis chemotherapy. Five days post-treatment, 43 patients received MV-MTX by intrapleural infusion every 48 h, six times over the course of 12 days, while the remaining 48 patients received saline placebo. Forty patients from the MV-MTX group and 39 patients from the placebo group were included in the final analysis. Pleural effusion volume was significantly decreased in the MV-MTX group compared with control. At 1 year post-treatment, survival was 77.5% for the MV-MTX group and 59.0% for the placebo group, although these differences were not statistically significant. Together, these data demonstrate EVs may serve as effective delivery vehicles of chemotherapeutic drugs for cancer treatment.

Treatment of COVID-19

MSCs are multipotent progenitor cells that differentiate into a variety of different cell types and support hematopoiesis through the release of a variety of molecules integral to the proliferation and differentiation of hematopoietic stem and progenitor cells.[165–167] Several clinical trials have demonstrated the efficacy of MSCs for treatment of COVID-19 symptoms, as MSCs can modulate the immune response and inhibit production of pro-inflammatory cytokines that can lead to a cytokine storm.[168–171] EVs isolated from MSCs retain many of the natural properties of MSCs,[172,173] and have been investigated as a cell-free alternative for treatment of COVID-19 symptoms. The safety and efficacy of exosomes derived from allogeneic bone marrow MSCs (designated ExoFlo) was examined in a clinical trial as a potential treatment for acute respiratory distress syndrome (ARDS) caused by severe COVID-19.[174] Unfortunately, details about how the exosomes were prepared were not included in the study. ExoFlos were not loaded with exogenous cargo, rather these exosomes were predicted to innately convey the regenerative and immunomodulatory properties of MSCs. Twenty-four patients were administered a one-time, 15-mL i.v. infusion of ExoFlo and monitored for 14 days. Seventeen of 24 (71%) patients recovered, three of 24 (13%) patients remained critically ill but stable, and four of 24 (16%) patients succumbed to COVID-19. These results can be compared with historical data, in which patients with COVID-19-induced ARDS experience a 39% mortality rate.[175] A significant decrease in C-reactive protein (CRP), ferritin, and D-dimer, and significant increase in oxygenation, lymphocyte counts, and neutrophil count was reported.[174] This pilot study was followed by a phase 2 placebo-controlled clinical trial in which ExoFlo was again administered to patients with ARDS caused by COVID-19.[176] A total of 102 patients were included in the study, and patients received an i.v. infusion of 100 mL of saline (n = 34), 90 mL saline with 10 mL of ExoFlo (n = 34), or 85 mL saline with 15 mL ExoFlo (n = 34) on day 1 and day 4, for a total of two treatments. The mortality rate at 60 days post-treatment was 48.4% in the placebo group, 46.6% in the ExoFlo 10 mL group, and 30.4% in the ExoFlo 15 mL group. This placebo-controlled study further demonstrated the efficacy of the 15-mL dose of ExoFlo for COVID-19-induced ARDS.

Another trial examined the safety and efficacy of allogeneic adipose tissue MSC-derived exosomes for treatment of COVID-19 pneumonia.[177] In this study, seven patients with severe COVID-19-induced ARDS were treated with nebulized MSC-derived exosomes for 5 days. All patients tolerated the treatment well, and different degrees of resolution of pulmonary lesions were observed, with four of seven (57%) patients attaining the most pronounced resolution as assessed by computed tomography image scores. One patient demonstrated complete resolution of pulmonary lesions following treatment. A similar study examined the safety of umbilical cord MSC-derived exosomes for treatment of COVID-19-induced pneumonia.[178] In this pilot study, seven patients with COVID-19-induced pneumonia were treated with nebulized MSC-derived exosomes, twice a day, over the course of 7–16 days. This small trial concluded that nebulization of MSC-derived exosomes promoted absorption of pulmonary lesions in mild cases of COVID-19-induced pneumonia.

A clinical trial compared the efficacy of MSC therapy and MSCs combined with MSC-derived EVs for treatment of COVID-19-induced ARDS.[179] One study group (n = 11) received two consecutive i.v. doses of umbilical cord or placental MSCs, while the other study group (n = 8) received one i.v. dose of the MSCs followed by one i.v. dose of 1,600 μg of MSC-derived EVs, both compared with a control group (n = 24). Forty-eight hours post-dosing, eight of 11 (72%) patients in the MSCs-alone group, eight of eight (100%) in the MSCs and MSCs + EVs group, and 16 of 24 (66%) patients in the control group were alive. Causes for mortality were reported to be multifactorial and attributed to several causes including acute ischemic cerebral stroke, congestive heart failure, multi-organ failure, myocardial infarction, pulmonary dysfunction, and septic shock. Changes in interleukin (IL)-6, interferon (IFN)-γ, and CRP were most pronounced in the MSCs + EVs group, while changes in TNF-α was most pronounced in the MSCs-alone group, and decrease in plasma IL-6 concentration was also noted in this treatment group. Partial thromboplastin time was significantly decreased in the MSCs-alone group, which is associated with a lower risk of thrombotic and disseminated intravascular coagulation.[179,180] The results from this

study suggest that MSCs and MSCs + MSCs-derived EVs are beneficial for treatment of COVID-19-induced ARDS, and the addition of MSC-derived EVs as part of a therapeutic regimen may allow for more robust changes in specific inflammatory cytokines.

Other therapeutics

EVs isolated from platelets (pEVs) have been used in a clinical trial to examine their potential as a treatment for delayed wound healing.[181] pEVs were shown to contain proteins essential in the wound-healing process, such as insulin growth factor and transforming growth factor β (TGF-β). In a double-blind, placebo-controlled clinical trial in healthy adults who had punch biopsy-induced wounds, injection of pEVs was shown to be safe and well tolerated. No difference in wound healing was observed between healthy and control participants. However, the healthy participants were not deficient in any aspect of wound healing and thus would be expected to undergo efficient, normal wound healing. A phase II study examining therapeutic efficacy in patients with disrupted or delayed wound healing is required to make a definitive conclusion about the utility of pEVs for this application.

Exosomes isolated from human placental MSCs have been explored as a possible treatment of perianal fistulas in multiple clinical trials.[182,183] In one study, 11 patients without Crohn's disease who had perianal fistulas for a minimum of 1 year that had persisted despite standard medical and surgical treatment were injected with MSC-derived exosomes along the fistula tract once a week for 3 weeks.[182] Patients were followed for 6 months post-treatment, and 10 of 11 patients (91%) showed improvement, and five of 11 patients (45%) demonstrated complete resolution of the fistulas. In a similar study, MSC-derived exosomes were injected along the fistula tract once a week for 3 weeks in five patients with Crohn's disease with perianal fistulas that had persisted for 6 months following TNF-α therapy. Patients were followed for 6 months post-treatment, and improvement was observed in four of five patients (80%), with three of five patients (60%) completely healed. Thus, the cargo innately carried within EVs derived from MSCs holds promise for treatment of a number of diseases.

CONCLUSION

Pre-clinical studies of EVs have investigated numerous engineering strategies to increase the loading of cargoes into EVs, including proteins, mRNAs, miRNAs, and siRNAs. Upon systemic delivery *in vivo*, EVs show a wide biodistribution, including to the brain, which suggests EVs may be a promising therapeutic vehicle for diseases of the central nervous system, an area of the body that has been historically difficult to reach. Success of EV-based therapeutics has already been demonstrated in early clinical trials. Thus, although the characterization and therapeutic use of EVs is still in its infancy, these particles have a strong potential to provide successful treatments for many different diseases.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.A.R.; literature search, C.A.R.; resources, R.J.P.; writing—original draft, C.A.R.; writing—review and editing, C.A.R. and R.J.P.; supervision, R.J.P.; funding acquisition, R.J.P.; project administration, R.J.P. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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