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RNA delivery by extracellular vesicles in mammalian cells and its applications

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Abstract | The term 'extracellular vesicles' refers to a heterogeneous population of vesicular bodies of cellular origin that derive either from the endosomal compartment (exosomes) or as a result of shedding from the plasma membrane (microvesicles, oncosomes and apoptotic bodies). Extracellular vesicles carry a variety of cargo, including RNAs, proteins, lipids and DNA, which can be taken up by other cells, both in the direct vicinity of the source cell and at distant sites in the body via biofluids, and elicit a variety of phenotypic responses. Owing to their unique biology and roles in cell–cell communication, extracellular vesicles have attracted strong interest, which is further enhanced by their potential clinical utility. Because extracellular vesicles derive their cargo from the contents of the cells that produce them, they are attractive sources of biomarkers for a variety of diseases. Furthermore, studies demonstrating phenotypic effects of specific extracellular vesicle-associated cargo on target cells have stoked interest in extracellular vesicles as therapeutic vehicles. There is particularly strong evidence that the RNA cargo of extracellular vesicles can alter recipient cell gene expression and function. During the past decade, extracellular vesicles and their RNA cargo have become better defined, but many aspects of extracellular vesicle biology remain to be elucidated. These include selective cargo loading resulting in substantial differences between the composition of extracellular vesicles and source cells; heterogeneity in extracellular vesicle size and composition; and undefined mechanisms for the uptake of extracellular vesicles into recipient cells and the fates of their cargo. Further progress in unravelling the basic mechanisms of extracellular vesicle biogenesis, transport, and cargo delivery and function is needed for successful clinical implementation. This Review focuses on the current state of knowledge pertaining to packaging, transport and function of RNAs in extracellular vesicles and outlines the progress made thus far towards their clinical applications.

Astrocytes
Glial cells of the central nervous system.

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Extracellular vesicles are membrane-enclosed nanoscale particles released from essentially all prokaryotic and eukaryotic cells that carry proteins, lipids, RNA and DNA. They can deliver information between cells through the extracellular space, with strong evidence for functional activity provided by their phenotypic effects on recipient cells. Cargo RNAs of extracellular vesicles include various biotypes that represent a selected portion of the RNA content of the source cell, with a strong bias towards small non-coding RNAs, although fragmented and intact mRNA, ribosomal RNA (rRNA) and long non-coding RNA (lncRNA) molecules can be found. Ample evidence supports the ability of RNAs enclosed in extracellular vesicles to impact the functional properties of cells that take them up (TABLE 1). For example, extracellular vesicles in plasma promote repair of cardiac cells¹, vesicles released by astrocytes contribute

to neurodegeneration² and tumour-derived vesicles create a favourable microenvironment for cancer progression³ and metastases⁴. However, given that the functions of many non-coding RNAs (ncRNAs) are incompletely understood and that delivery of any given extracellular vesicle-associated RNA is accompanied by delivery of multiple other biomolecules, the complex language of extracellular vesicle-mediated communication remains to be deciphered. Moreover, the cellular mechanisms involved in the trafficking and fate of extracellular RNAs (exRNAs), including the possibility of packaging different RNA sequences into different types of extracellular vesicles, differential uptake into specific recipient cell types and fate on uptake are still being elucidated (FIG. 1).

The RNA contained in extracellular vesicles reflects the type and the physiological/pathological state of the source cells, but differs substantially from the cellular

Table 1 | **Functional delivery of miRNAs by extracellular vesicles**

Source cell	RNA	Target cell	Effect	Refs
Rhabdomyosarcoma	miR-486-5p	Mouse embryonic fibroblasts, C2C12 immortalized mouse myoblast cell line, immortalized myoblasts	Cell migration, invasion, colony formation (in vitro)	258
Glioblastoma	miR-9 miR-21	Brain endothelial cells	Angiogenesis (in vitro)	3,259
		Microglia	Immunosuppression (in vivo)	
Breast cancer cells	Various	Epithelial cells	Promote tumorigenesis, invasion, cell proliferation (in vitro)	260
		Endothelial cells	Promote angiogenesis (in vitro)	
		Breast cancer cells	Increase drug resistance (in vitro)	
	miR-9, miR-195, miR-203	Cancer stem cells	Increase expression of stemness genes (in vitro and in vivo)	261
Cardiac progenitor cells	miR-210, miR-132, miR-21, miR-451, miR-146a	Various	Inhibit apoptosis, promote angiogenesis, improve cardiac function, inhibit myocardial fibrosis (in vitro and in vivo)	Reviewed in ²⁶²
Cardiomyocytes	miR-320	Various cell types	Inhibit angiogenesis (in vivo)	Reviewed in ²⁶³
	miR-30a		Regulate autophagy (in vitro)	
	miR-29b, miR-455		Inhibit fibrosis (in vivo)	
	miR-27a, miR-28-3p, miR-34a		Contribute to oxidative stress (in vitro)	
	miR-208a		Promote fibrosis (in vivo)	
Neuroprecursor cells	miR-21a	Neural progenitor cells	Promote neurogenesis (in vitro)	264
Adipose macrophage	miR-155	Bone marrow mesenchymal stem cells	Increase insulin resistance (in vitro and in vivo)	265
Adipose mesenchymal stem cells	miR-375	Bone marrow mesenchymal stem cells	Osteogenic differentiation, enhance bone regeneration (in vitro and in vivo)	266
Adipocytes (from brown adipose tissue)	miR-99b	Liver	Increase <i>Fgf21</i> expression, increase glucose tolerance (in vivo)	267

miRNA, microRNA.

Large oncosomes

Extracellular vesicles between 1,000 nm and 10,000 nm in diameter derived from tumour cells that contain abnormal and transforming macromolecules, in addition to other cargo.

RNA content, in terms of both the types of RNA and the relative concentrations of specific RNA sequences. The extracellular vesicle populations carried in biofluids, tissues and conditioned medium from cultured cells are heterogeneous with respect to size, morphology and composition. Four major subclasses of extracellular vesicles appear to arise from distinct biogenesis pathways and can be distinguished roughly in the basis of size: exosomes (50–150 nm), microvesicles (100–1,000 nm), large oncosomes (1,000–10,000 nm) and apoptotic bodies (100–5,000 nm), but are difficult to distinguish from high-density and low-density lipoproteins, chylomicrons, protein aggregates and cell debris⁵. Guidelines for

standardization of terminology, methods and reporting are being developed to improve experimental reproducibility across studies^{6,7}. The size of most extracellular vesicles (which also limits the number of cargo molecules/vsicles) places them below the resolution and sensitivity thresholds of standard light microscopy and fluorescence-activated sorting techniques. Overlap in the sizes and other biophysical properties among different extracellular vesicle subclasses and lack of known unique markers for each subclass^{8,9} have made it difficult to define the cargo (including RNAs) of different subclasses with confidence⁵. Technical factors, including the use of different methods for isolation of extracellular vesicles and their RNA, can strongly influence RNA profiling results (see, for example, REFS^{10–16}). Separation of RNA in vesicles from RNAs associated with other exRNA carriers, including lipoproteins¹⁷ and ribonucleoproteins¹⁸, is also challenging (see REFS^{5,6,10,17,18} and the [exRNA Atlas](#)¹¹). A variety of approaches have been used to address these concerns, including culture of cells in serum-free medium (to avoid contamination with serum-derived extracellular vesicles) and separation of extracellular vesicle subclasses and other exRNA

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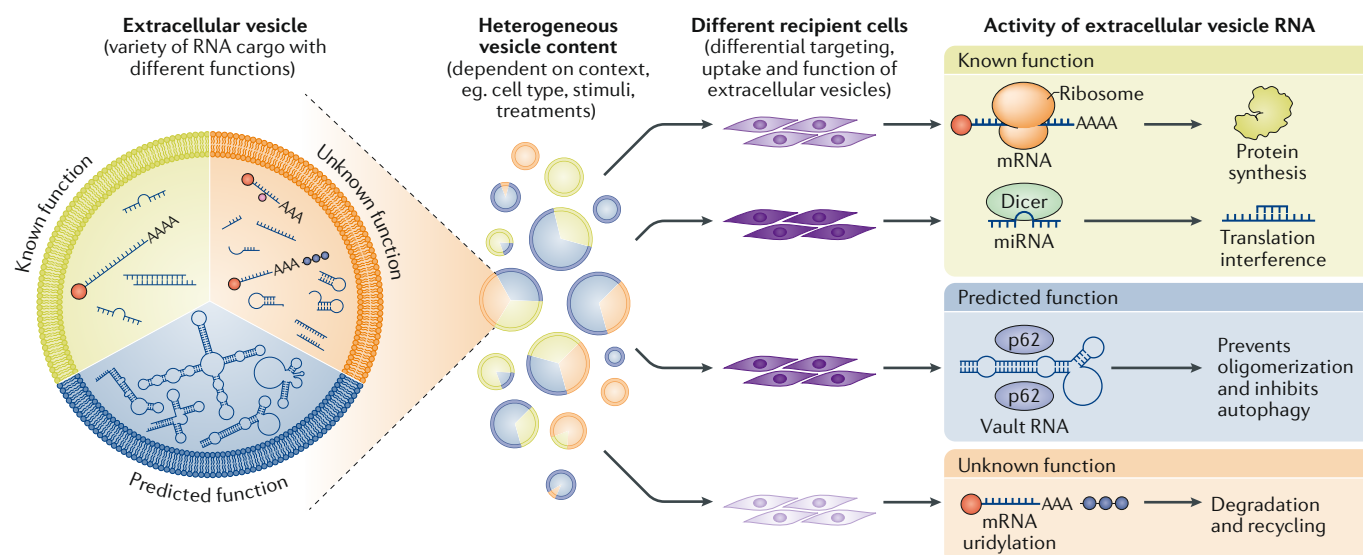


Fig. 1 | Principles of functional cell communication by extracellular vesicle RNA. Extracellular vesicles are generated as highly heterogeneous populations with different types of RNA cargo within them and in different amounts and proportions. Functionally, these RNAs can be divided into those with known functions, for example some mRNA, microRNA (miRNA) and small interfering RNA (green zone), those with predicted functions, for example, some transfer RNA, small nucleolar RNA, small nuclear RNA, Y RNA and vault RNA (blue zone) and those with unknown functions, for example, fragmented and degraded (methylated and uridyldated) RNA species (orange zone). This heterogeneity is further enhanced by the fact that extracellular vesicle cargo content strongly depends on the context (for example, cell type, stimuli and treatments). The effect that different kinds of RNA in vesicles can have on recipient cells is dictated in part by the nature of these cells, which will show differential capability for recognizing specific vesicles, their uptake and ultimately their functional effect.

Lipoproteins

Macromolecular aggregates of proteins (apoproteins) and different classes of hydrophobic and amphipathic biomolecules that range in size from 10 nm to 1 μ m. These aggregates have the function of collecting and transporting lipids, triglycerides, free cholesterol (unesterified cholesterol) and esterified cholesterol in blood plasma.

Ribonucleoproteins

Proteins structurally associated with nucleic acids, the prosthetic group (nucleic acid) of which contains ribose.

High-resolution density gradient centrifugation

A method in which extracellular vesicles and particles are centrifuged in a gradient medium (for example, iodixanol) and separated primarily on the basis of their density.

Size-exclusion chromatography

A technique in which a porous stationary phase is used to sort extracellular vesicles from proteins on the basis of the larger size of the former.

carriers by high-resolution density gradient centrifugation¹⁰, size-exclusion chromatography¹⁹, asymmetric field-flow fractionation^{20,21} and immunoaffinity purification⁹.

In addition to serving as a novel mode of communication among cells, RNAs in extracellular vesicles can also serve as biomarkers and therapeutics for a variety of diseases. Since extracellular vesicles have been found in all tested biofluids, many of which can be collected non-invasively, the RNAs within them can be interrogated in real time to evaluate the functional state of a range of cell and tissue types²². The potential of extracellular vesicles to protect nucleic acids and other biological macromolecules from degradation in vivo, and to deliver them to recipient cells in a targeted manner without immune activation, makes them an exciting new modality for delivery of RNA therapeutics, including small interfering RNAs (siRNAs), microRNAs (miRNAs), antisense oligonucleotides, mRNAs, guide RNAs and self-amplifying RNAs.

In this Review, we discuss the biogenesis, biological functions and emerging clinical applications of extracellular vesicle RNAs in mammalian cells. Throughout, we highlight the key open questions and technical challenges that currently limit the broader translational use of extracellular vesicles. We are optimistic that these hurdles will be overcome soon, allowing better understanding and control of extracellular vesicle-mediated delivery of RNAs to specific cellular targets and opening up new opportunities for the detection and treatment of various diseases, including cardiovascular, neurodegenerative and metabolic diseases and cancers.

Extracellular vesicles as RNA carriers

Extracellular vesicles carry within them a wide variety of RNA sequences representing many biotypes of RNA. This diversity of RNAs, as well as the small size and heterogeneity of extracellular vesicles and the overall low concentration of RNA in extracellular vesicles, has complicated characterization of the RNA cargo of extracellular vesicle subclasses (BOX 1; TABLE 2).

Extracellular vesicle RNA content. To understand intercellular communication mediated by extracellular vesicle RNAs, it is important to develop a comprehensive catalogue of the types of RNAs and sequences within them. Initial studies reported that extracellular vesicles contained mRNAs and mature miRNA sequences, as well as ncRNAs, with a peak size of 200 nucleotides, but extending out to 5 kb or more^{23–25}. Since then, more comprehensive studies have found the majority of known ncRNA biotypes, including small nuclear RNAs, small nucleolar RNAs (snoRNAs), rRNAs, lncRNAs, PIWI-interacting RNAs (piRNAs), transfer RNAs (tRNAs), mitochondrial RNAs, Y RNAs and vault RNAs (vtRNAs)^{26–28}. miRNA precursors (pre-miRNAs) have also been reported in tumour microvesicles, along with the RNA interference processing machinery components Dicer and Argonaute^{29–31}. However, the methods used in these studies did not separate extracellular vesicles from free ribonucleoproteins¹⁶ or lipoproteins³². Moreover, among the many studies using proteomic methods to analyse the composition of extracellular vesicles, only one has reported the presence of Argonaute 2 (AGO2)³³, and none has detected Dicer. One study that used density

Asymmetric field-flow fractionation

A technique that separates differentially sized particles ranging from 1 nm to 100 μ m in a fluid suspension where the rate of laminar flow is not uniform. A force is applied in a perpendicular direction, facilitating the separation of particles on the basis of size.

Immunoaffinity purification

A method of isolation of extracellular vesicles using antibodies, typically conjugated to magnetic beads, that recognize proteins exposed on the surface of extracellular vesicles.

Antisense oligonucleotides

Sequences of DNA or RNA roughly 15–25 nucleotides in length that hybridize to complementary RNA targets, resulting in altered splicing or degradation of the targeted RNA.

Guide RNAs

RNAs with a specific sequence that recognize and aid nucleases, such as Cas9, in cutting the genomic DNA region of interest for targeted genome engineering.

Self-amplifying RNAs

Engineered viral genomes encoding proteins involved in the replication mechanism and therefore capable of directing their own replication.

Small nuclear RNAs

Short sequences (about 150 nucleotides) of ribonucleic acid, usually very rich in uracil, involved in the maturation of mRNA in the cell nucleus.

Small nucleolar RNAs

(snoRNAs). RNA species consisting of 60–300 nucleotides capable of promoting certain chemical modifications and maturation of many cellular RNAs.

PIWI-interacting RNAs

(piRNAs). RNAs approximately 21–35 nucleotides long representing a class of RNAs that form protein–RNA complexes by interacting with PIWI proteins. These piRNA complexes have been linked to the transcriptional silencing of retrotransposons and other genetic elements in germ line cells.

Box 1 | Technical considerations in characterization of extracellular vesicle RNA cargo

Different RNA isolation and measurement methods will produce different extracellular RNA (exRNA) profiles from the same biofluid or RNA sample^{9,11,16,24,112,285–291} owing to:

- unique properties of each exRNA carrier type, such as susceptibility to different chemical disruption methods
- biases of each RNA isolation and profiling method

Quantification of the stoichiometry of extracellular vesicle cargo is difficult because:

- there are low numbers of cargo molecules per vesicle. On average, only one microRNA per extracellular vesicle³⁵ to one microRNA per 100 extracellular vesicles²⁹² and one intact long RNA molecule (for example, a full-length mRNA) per 1,000 extracellular vesicles¹¹²
- most sensitive methods (quantitative reverse transcription–PCR and Droplet Digital PCR) are tailored to specific RNA species. However, different RNA sequences can differ greatly in abundance, so it is difficult to accurately extrapolate measurements obtained for one specific RNA to other RNAs
- RNA biotypes are not evenly distributed among the different extracellular vesicle subtypes^{112,135}
- concordance among existing methods for quantification of extracellular vesicles is poor^{293–295}
- single-vesicle characterization methods (for example, through simultaneous visualization of extracellular vesicle protein markers and RNA cargo by super-resolution microscopy²⁹⁶) are needed. Current methods are targeted and of low throughput

Different strategies can be applied to allow profiles from different biological groups (for example, cases and controls for a given disease) to be reliably compared:

- use the same method throughout a study. Intramethod reproducibility of many exRNA isolation and analysis methods is quite high, so consistent use of the same method allows differential expression studies to be performed
- use computational approaches to separate (or deconvolute) the data from a mixed population of exRNA carriers into profiles of the component carrier subclasses¹¹

Candidate biomarkers and signalling RNAs identified by comprehensive exRNA profiling methods should be validated:

- biomarkers should be validated in multiple independent study populations
- candidate signalling RNAs should be functionally validated by multiple approaches

gradient ultracentrifugation to isolate extracellular vesicles from cell culture supernatant of cancer cells did convincingly demonstrate that overexpression of the *KRAS* oncogene promotes sorting of AGO2-associated miRNAs into extracellular vesicles³⁴. Thus, whether pre-miRNAs and their processing machinery are transferred via extracellular vesicles in normal cells remains to be established. Finally, extracellular vesicle-associated circular RNAs have also been detected by use of specific data analysis pipelines that can identify back-spliced transcripts^{35–40}.

In general, RNA in extracellular vesicles can be categorized into three types: RNAs that have been established to be functional when carried by extracellular vesicles between cells, such as intact mRNAs and miRNAs; intact RNAs that are predicted to be functional but have not been definitively demonstrated to mediate intercellular communication (for example, piRNAs and vtRNAs); and fragments of RNAs (for example, tRNA fragments and fragments of mRNAs and rRNAs), some of which may be functional but others may be non-functional degradation products (FIG. 1).

Specific enrichment of RNAs in extracellular vesicles.

The transcriptomes of different cell types are partially reflected in their extracellular vesicle RNA cargo^{14,41}, but the RNA profiles of extracellular vesicles differ substantially from those of their cells of origin^{24,42–44}, suggesting that some RNA species are selectively incorporated into extracellular vesicles. A recent small RNA-sequencing study focusing on 17–35 nucleotide sequences from extracellular vesicles across five cell lines found that rRNA fragments accounted for 30–94% of the reads

and ‘small RNAs’ accounted for 2–40% of the reads, depending on the cell type⁴¹. In the ‘small RNA’ category, approximately 15% in some lines and nearly 80% in others mapped to miRNAs, which inversely correlated with the abundance of piRNAs (ranging from 80% to 20%). Notably all of the cell lines included in that study were highly aneuploid, and therefore the results might not reflect the RNA composition of extracellular vesicles in cells with normal genomic composition.

In addition to these differences in RNA biotypes, specific RNA sequences are packaged into extracellular vesicles with different efficiencies. Early studies focused on preferential loading of specific miRNAs into extracellular vesicles^{45,46}, and a recent study found that during T cell activation, tRNA fragments are preferentially enriched in extracellular vesicles compared with miRNAs⁴⁷. The physiological states of cells can also impact exRNA profiles, including oxidative stress⁴⁸, pain⁴⁹ and exercise^{50–52}, all of which alter levels of specific extracellular miRNAs in conditioned medium from cultured cells, as well as in patient serum/plasma.

Packaging of extracellular vesicle RNA

Biogenesis of extracellular vesicles is relatively well studied, and several modes and molecular components that drive the process have been established^{5,53} (FIG. 2). Exosomes are generated by invagination of the endosomal membrane forming intraluminal vesicles within multivesicular bodies (MVBs). This process involves participation of the endosomal sorting complex required for transport, as well as RAB27A/RAB27B, tumour susceptibility gene 101 protein (TSG101), ALIX, vacuolar protein sorting-associated protein 4 (VPS4)⁵ and

Y RNAs

Small non-coding RNAs essential for the initiation of chromosomal DNA replication. When bound to the RO60, they are involved in RNA stability and cellular responses to stress.

Vault RNAs

(vtRNAs). Small RNA components of the vault ribonucleoprotein complex. Approximately 100 nucleotides in length, they function as the intracellular and nucleocytoplasmic transporters.

Dicer

The RNase III known as Dicer is involved in the cleavage of double-stranded RNA and microRNA precursor molecules forming short double-stranded fragments called 'small interfering RNAs' or 'microRNAs'.

Argonaute

The Argonaute protein family has a critical role in the RNA-induced silencing complex. These proteins form complexes with microRNAs, small interfering RNAs and PIWI-interacting RNAs, interfering with mRNA translation and/or degrading target mRNAs.

Circular RNAs

Single-stranded, non-coding RNAs that may play a role in transcriptional regulation and in mediating protein interactions. They are characterized by a covalently closed loop feature without 5'-end caps or 3' poly(A) tails.

tRNA fragments

Transfer RNA (tRNA)-derived fragments are cleaved from either mature or precursor tRNAs. Functionally, they can act through RNA interference pathways, participate in the formation of stress granules, move mRNA from RNA-binding proteins and/or inhibit translation.

Multivesicular bodies

(MVBs). Represent a late endosomal compartment, which contains luminal vesicles. Vesicles within the MVBs either can be degraded when MVBs fuse with lysosomes or can be secreted as exosomes when MVBs fuse with the plasma membrane.

Table 2 | Challenges and limitations for extracellular vesicle and exRNA research

Challenge/limitation	Consequences	Potential solutions
Heterogeneity of extracellular vesicles and their (RNA) cargo (differences between cell types, influence of external conditions, additional stimuli, etc.)	Lack of concordance among studies using different cell types or biofluid sources Inability to determine which mechanisms/features of extracellular vesicle biogenesis and cargo selection are universal and which are cell type specific Inability to precisely manipulate the cargo content for improved transfer of that content	Systematic studies of extracellular vesicle biogenesis and cargo content including multiple diverse cell types
Sample-to-sample heterogeneity in extracellular vesicle and other exRNA carriers	High sample-to-sample technical variability that is not related to the biological variable of interest	Develop methods to determine the relative abundance of each carrier subclass in individual samples
Differences (efficiency, purity, throughput, etc.) in methods for isolating extracellular vesicle carriers	Lack of concordance among studies using different extracellular vesicle and exRNA isolation techniques Difficulties in determining exRNA activity on transfer	Develop methods for unbiased exRNA isolation Establish a comprehensive catalogue of extracellular vesicle and non-extracellular vesicle exRNA carriers Develop methods to isolate each carrier and characterize its cargo, and to perform single-extracellular-vesicle cargo analysis
Differences in sensitivity, specificity, reproducibility and bias for different RNA profiling methods	Lack of concordance among studies using different exRNA profiling techniques	Develop methods for unbiased exRNA profiling Establish standards that can be used to compare sensitivity, specificity and bias among methods

exRNA, extracellular RNA.

lipid microdomains (lipid rafts)⁵⁴. When MVBs fuse with the plasma membrane, the intraluminal vesicles are released into the extracellular space as exosomes. Exosome release relies on the cytoskeleton, such that MVBs are transported to the plasma membrane via microtubules⁵⁵, where branched actin, polymerized by Arp2/3 and stabilized by the actin-binding protein cortactin, supports their docking⁵⁶. Microvesicles bud out from the plasma membrane directly and share many of the same proteins involved in exosome biogenesis^{5,20}, which makes it challenging to experimentally dissect and manipulate the different extracellular vesicle carriers. In addition to these two basic modes of extracellular vesicle biogenesis, oncosomes form by blebbing off the plasma membrane of tumour cells. These extracellular vesicles are usually larger than microvesicles and are typically associated with cell motility^{57–60}. Release of oncosomes involves recruitment of the actin-bundling protein fascin, which together with the actin–plasma membrane crosslinker ezrin and the cell-surface glycoprotein podocalyxin promotes extrusion and scission of the plasma membrane²⁹. Other large extracellular vesicles, overlapping in size with oncosomes, form during apoptotic cell death when plasma membrane blebs and spike-like protrusions from the plasma membrane (microtubule spikes) break off during apoptosis (forming apoptopodia)^{5,60}. Finally, some extracellular vesicles can be generated with the engagement of proteins of viral origin, such as retroviral-like Gag proteins on the inner plasma membrane that

bind RNA and bud off from the cell membrane as virus-like particles.

During extracellular biogenesis, the cell's vesiculation machinery packages multiple RNA species into different subclasses of extracellular vesicles, contributing to a substantial portion of the exRNA pool⁶¹. Small size, high abundance, ability to associate with membranes and cytoplasmic (versus nuclear) location favour incorporation of a given RNA into extracellular vesicles. Most RNAs transit from the nucleus to specific cellular locations in association with RNA-binding proteins (RBPs), which can coalesce into large ribonucleoprotein particles (containing hundreds of different RNAs and many RBPs) that travel along the cytoskeleton^{62,63}. Of note, there are more than 500 RBPs in mammalian cells⁶⁴, and RBPs make up about 25% of the protein content of extracellular vesicles⁴¹. Information is lacking about the distribution of these RBPs in different extracellular vesicles and their role in loading RNA into these vesicles.

Multiple mechanisms have been implicated in RNA packaging into extracellular vesicles, including specific RNA sequence motifs and/or secondary configuration^{65,66}, differential affinity for membrane lipids^{67–69} and association with RBPs, such as AGO2 (REF.³⁴), ALIX⁷⁰, annexin A2 (REFS^{71,72}), major vault protein (MVP)^{73,74}, HuR⁷⁵, heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2B1) (REF.⁷⁶), YBX1 (REFS^{77–79}), SYNCRIP^{80,81}, lupus La protein⁸² and Arc1 (REF.⁸³) (FIG. 2). Two RBPs, heterogeneous nuclear ribonucleoprotein K (HNRNPK) and scaffold-attachment factor B1 (SAFB), regulate the

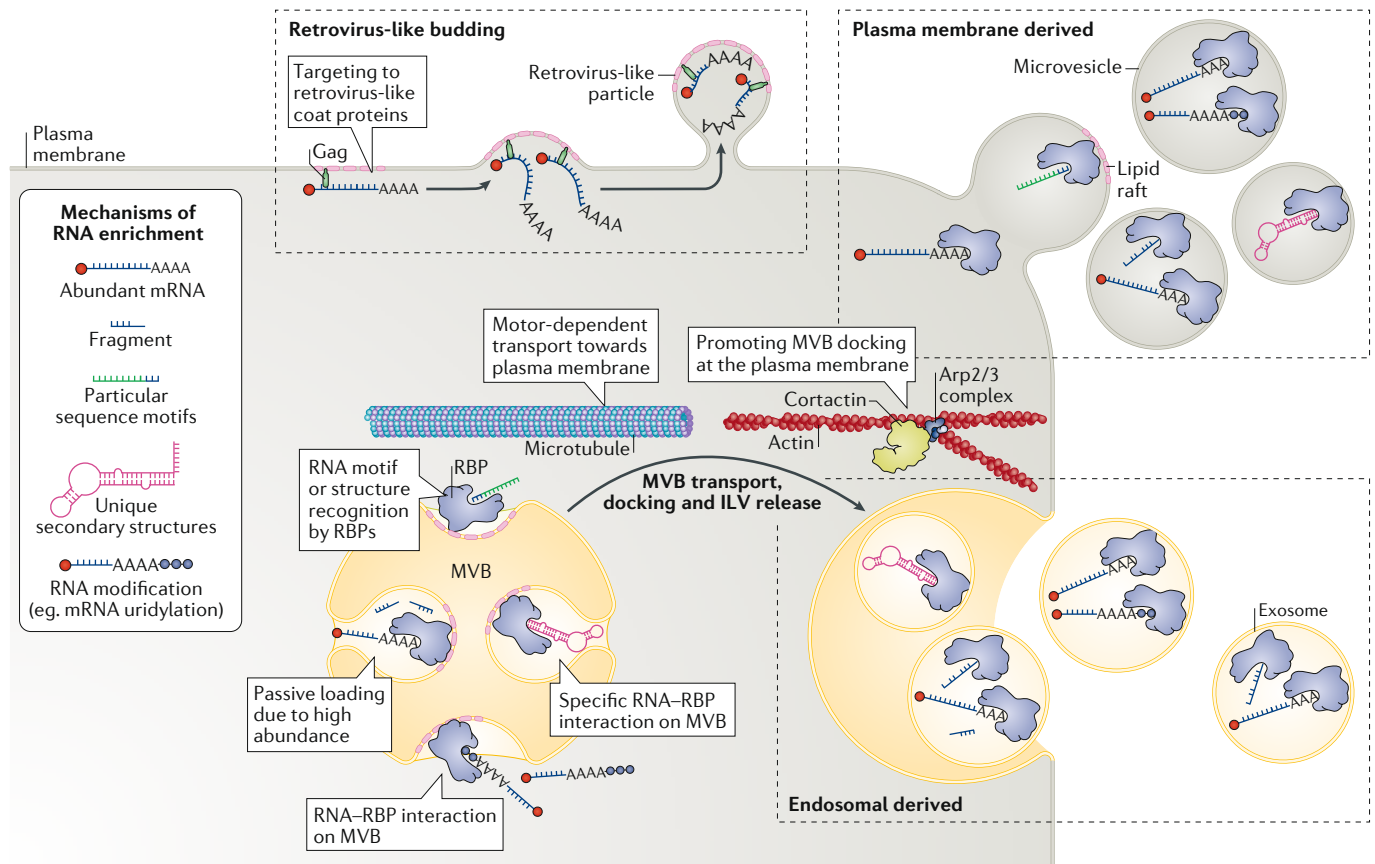


Fig. 2 | RNA packaging into extracellular vesicles and their release into the extracellular space. A variety of different RNA species can be packaged into extracellular vesicles. A number of modalities have been proposed for incorporation of (specific) RNAs into extracellular vesicles. First, RNAs can be targeted to the plasma membrane and released as microvesicles. They can also be targeted to the endosomal compartment and incorporated into intraluminal vesicles (ILVs) of the multivesicular body (MVB), which then can be targeted to the plasma membrane, where it fuses to release ILVs as exosomes. Both these modes of biogenesis share many factors, and hence the vesicle type and vesicle origin are typically difficult to ascertain and control. Membrane microdomains (lipid rafts) have been strongly associated with the release of extracellular vesicles. Also, cytoskeletal components are implicated in extracellular vesicle biogenesis, in particular for exosomes, which are transported via microtubules, and their docking at the plasma membrane is supported by Arp2/3-generated branched-actin filaments stabilized by the actin-bundling activity of cortactin. Cortical actin remodelling is also an important event in membrane shaping during microvesicle release (not shown). Loading of RNA into extracellular vesicles can occur via multiple routes: passively due to an abundance of the RNA in the cytosol; by recognition via a number of RNA-binding proteins (RBPs), such as Argonaute, annexin A2, major vault protein (MVP), heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2B1), YBX1, SYNCRIP and lupus La protein, that bind particular sequence motifs in the RNA or that recognize unique secondary RNA structures; and through specific modifications, such as uridylation. Packaging of RNA into extracellular vesicles can also be promoted by its recognition by retroviral coat proteins such as Gag (and their silent copies present in animal genomes), which efficiently target RNAs they recognize to the plasma membrane (or the membrane of the MVB; not shown), resulting in virus-like particle release.

Lipid rafts

Subdomains of the plasma membrane represented by accumulations of proteins and lipids, with high concentrations of cholesterol and glycosphingolipids.

Blebbing

A bulging of the plasma membrane accompanied by membrane decoupling from the underlying cytoskeleton, which has the potential to release vesicles surrounded by the plasma membrane.

small non-coding RNA composition of extracellular vesicles via secretory autophagy⁸⁴. Different RBPs display binding preferences for different RNA sequence motifs, but a full understanding of these binding interactions has yet to be established (see, for example, REFS^{71,76,80,85–87}). Other extracellular vesicle sorting signals may include RNA and/or RBP modifications, such as ubiquitylation, sumoylation, phosphorylation and uridylation, which also impact RNA splicing, stability and translation and miRNA biogenesis⁸⁸. Exosome biogenesis and miRNA-mediated gene silencing may be functionally linked^{89,90}. The small GTPase ARF6 appears to be involved in targeting of pre-miRNAs to oncosomes along with miRNA processing machinery²⁹.

Thus, many mechanisms can influence packaging of RNAs into extracellular vesicles, but the extent to which these mechanisms are specific to selected RNA species and how RNA cargo loading is regulated by the cells remain elusive.

Extracellular vesicle RNA delivery

A range of mechanisms have been elucidated for extracellular vesicle uptake by recipient cells; however, what dictates how particular cells respond to specific extracellular vesicles remains unknown². While uptake of vesicles is relatively well characterized, more work is needed to reveal the mechanisms by which extracellular vesicles deliver functional RNA to recipient cells.

Extracellular vesicle interactions with recipient cells.

Interaction of extracellular vesicles with recipient cells depends on the specific proteins, lipids and glycans on their surfaces, as well as their overall negative charge^{14,91}. Uptake of extracellular vesicles can be both selective and non-specific^{5,53}. For example, extracellular vesicles from tumour cells may home to different tissues by virtue of the expression of specific integrins on their surface⁵³, thereby setting up a tissue-specific niche for incoming metastatic tumour cells⁴. Once they are in contact with recipient cells, multiple routes of extracellular vesicle uptake have been reported, including clathrin/caveolin-mediated endocytosis, macropinocytosis, phagocytosis, lipid raft-mediated uptake or direct membrane fusion⁹² (FIG. 3). There are also reports of direct cell-to-cell extracellular vesicle transfer via tunnelling nanotubes for RNA delivery^{93–95}. The current focus of extracellular vesicle uptake is on endocytic pathways, which may result in part from the difficulty in assessing fusion events. Extracellular vesicles may also remain bound to cell surfaces without internalization (FIG. 3), engaging in cell signalling and antigen presentation⁵³. It is currently unknown whether cells control the interaction and uptake of extracellular vesicles depending on their subtype and/or physiological state.

Extracellular vesicle uptake and RNA release into recipient cells.

For functional activity of RNAs contained in extracellular vesicles within recipient cells, the RNAs need to enter the cytoplasm, and if the RNA enters through the endosomal pathway, the RNA must 'escape' from it (FIG. 3; Supplementary Table 1). Uptake of extracellular vesicles can be promoted at the cell surface. For example, binding to heparan sulfate proteoglycans on the plasma membrane increased uptake of tumour-derived extracellular vesicles via endocytosis^{96,97}, and specific integrin receptors can drive selective uptake of extracellular vesicles by certain primary tumours⁹⁸. Uptake of extracellular vesicles was also promoted by the interaction of T cell immunoglobulin and mucin domain-containing protein 4 (TIM4) molecules with phosphatidylserine present on extracellular vesicle membranes⁹⁹. Accordingly, these different types of molecules could be engineered on the surface of extracellular vesicles to promote their uptake⁹⁹.

Importantly, extracellular vesicle uptake through the endosomal and engulfment (phagocytosis and macropinocytosis) pathways poses challenges to delivery of functional RNA into the cytoplasm, as a prominent role of these pathways is to shuttle cargo to the lysosome for degradation¹⁰⁰. Indeed, it has been demonstrated that despite successful uptake, generation of functional protein from extracellular vesicle-derived mRNA in recipient cells was negligible, suggesting that endosomal escape is critical to RNA function¹⁰¹. It is possible that extracellular vesicles might naturally incorporate some mechanisms for endosomal escape, which so far remain unknown. Nevertheless, establishing reliable means of increasing the efficiency of extracellular vesicle cargo escape from the endosomal compartment will likely be essential to widen the application potential of extracellular vesicles (see also later).

Progress on understanding extracellular vesicle cargo delivery to recipient cells has been hampered by the lack of sensitive and specific markers for optical and molecular tracking. Protein delivery can be monitored by use of a GFP–luciferase dual split tag with a fluorescent and bioluminescent readout when extracellular vesicle cargo is united with its other half in the cytosol¹⁰². Tools developed for tracking of nanoparticles, liposomes and viruses may prove useful in elucidating these mechanisms (TABLE 3; Supplementary Table 1). RNA cargo delivery could be facilitated by use of recipient cells null for the RNA being tracked, but this experimental manipulation might alter the cell phenotype, thereby skewing interpretation^{3,103}.

Biology of extracellular vesicle RNA

Once the extracellular vesicle RNA cargo has been delivered to the recipient cell and has escaped the degradative pathway, it may elicit a functional response. Among the various RNA biotypes present in extracellular vesicles, most is known about miRNAs as mediators of cell–cell communication, with more limited information on the functions of other RNA biotypes. Several difficulties arise in establishing the functionality of RNA in extracellular vesicles. First, overexpression systems that increase the amounts of a particular RNA in extracellular vesicles can result in supraphysiological levels of the RNA in source cells, potentially resulting in changes in the extracellular vesicle release process and/or altering levels of other cargo. Functional effects of the RNA cargo of interest may be affected by the other cargo delivered at the same time. The population of exRNAs — importantly RNA in extracellular vesicles — in conditioned cell culture medium is highly dependent on the culture methods used. Most prominently, bovine serum, which is widely used as a medium supplement, contains bovine RNA in extracellular vesicles and non-vesicular entities. These exRNAs can confound molecular and functional readouts in the extracellular vesicle–recipient cell, particularly as many miRNAs are highly conserved among mammalian species¹⁰⁴. Methods to remove exRNAs from bovine serum through ultracentrifugation or affinity purification result in partial, but not complete, depletion^{12,13}. Another confounding problem is mycoplasma infection of cell cultures¹⁰⁵, and this must be monitored frequently.

Extracellular vesicle RNA cargoes can be divided on the basis of knowledge of their bioactivity into those with known functions, those with predicted functions and those with unknown functions (FIG. 1), with the third category evoking the early concept of extracellular vesicles as disposal units for some molecules^{16,106}. Nevertheless, even when used as a means for discarding, RNA may have functional effects on both donor and recipient cells. Several prominent examples of functional effects for different species of extracellular vesicle RNA are discussed in the following sections.

miRNAs. Numerous examples of extracellular vesicle-mediated functional transfer of miRNAs have been demonstrated with use of both *in vitro* and *in vivo* models for a variety of diseases and physiological

Secretory autophagy

The transportation of proteins via the autophagosome to the plasma membrane, multivesicular bodies or secretory lysosomes resulting in extracellular release of proteins.

Macropinocytosis

An endocytic process that results in the cellular uptake of fluid and particles less than 0.2 µm in diameter via invaginated membranes that then form intracellular vesicles.

Tunnelling nanotubes

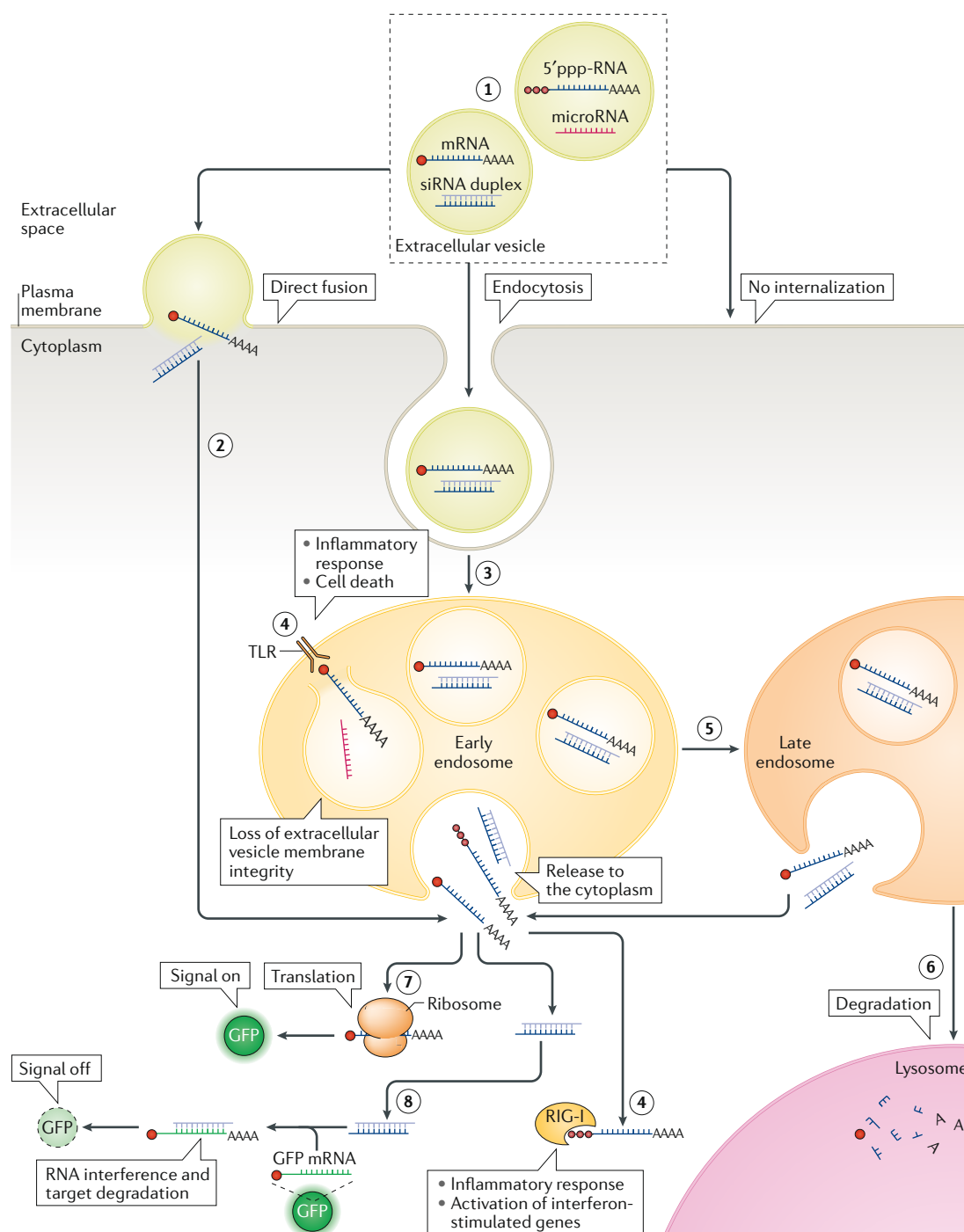
Dynamic actin-driven protrusions from the plasma membrane of cells that form connections between cells more than 100 µm away to facilitate intercellular communication.

Proteoglycans

Highly glycosylated proteins that form the major component of the extracellular matrix. Glycosaminoglycans of proteoglycans are divided into seven main classes: hyaluronic acid, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, keratan sulfate I, keratan sulfate II and heparin. Proteoglycans act as molecular filters, regulating movement of macromolecules, such as albumin and immunoglobulins, throughout the matrix.

Liposomes

Small spherical nanoparticles that contain an aqueous core solution and at least one phospholipid bilayer. They can be prepared from biological membranes and are often used for drug delivery.



Amyloid precursor protein
A transmembrane protein that is a precursor of β -amyloid, a protein involved in the pathology of Alzheimer disease.

Mesenchymal stem cells
Multipotent stromal progenitor cells found in a number of different tissues that are capable of self-renewal and differentiation into a variety of cell types.

states with a broad range of downstream effects. For example, miR-193b is secreted via extracellular vesicles in the central nervous system and decreases levels of amyloid precursor protein in neuronal cells, providing reductions in the pathology of Alzheimer disease, and the content of this miRNA in extracellular vesicles can be used as a biomarker for this disease¹⁰⁷. In other examples in the nervous system, neurons produce miR-124a-loaded extracellular vesicles that regulate the glutamate transporter in astrocytes, which is important for synaptic transmission¹⁰⁸. In the context of brain cancer, astrocytes secrete extracellular vesicles with miR-19, which inhibits an important tumour

suppressor, PTEN, in cancer cells, thereby promoting growth of brain metastases¹⁰⁹. As another example, extracellular vesicles from mesenchymal stem cells enriched in miR-375 via its overexpression stimulated bone regeneration by bone marrow stem cells¹¹⁰. Two critical miRNAs that regulate inflammation, miR-155 and miR-146a, are exchanged between dendritic cells in vivo, with the latter inhibiting and the former promoting endotoxin-induced inflammation in mice¹¹¹. Other recent examples of transfer of functional miRNAs by extracellular vesicles from cancer, cardiac, neural, immune and adipose cells are summarized in TABLE 1.

◀ **Fig. 3 | Extracellular vesicle RNA cargo interaction with recipient cells and its functional delivery.** After encountering the recipient cell, the extracellular vesicle is typically bound to its surface via cell-surface receptors (although extracellular vesicles can also be engulfed from the environment in a process known as macropinocytosis; not shown). After establishing an interaction with the cell surface, the vesicle can remain bound on the surface or can be internalized (1). One possible means of internalization is direct fusion with the plasma membrane (2), but the most common mechanism of internalization involves endocytosis, whereby extracellular vesicles are taken up to endosomes (3). In the endosome, RNA content might be released into the luminal space (if the integrity of the extracellular vesicle membrane is perturbed) or it might be released into the cytoplasm (of note, the frequency of these events is low, and endosomal escape of extracellular vesicle cargo is currently an important bottleneck in functional RNA cargo delivery by extracellular vesicles). In both cases RNAs can be recognized by pattern recognition receptors, such as Toll-like receptors (TLRs) and RIG-I or NOD-like receptors that reside in the endosome and in the cytoplasm, respectively, raising innate immune response signalling (4). Early endosomes will gradually transform into late endosomes with progressive internal acidification and possible release of RNA (stimulated by the decreasing pH) (5). Further down the endocytic pathway, endosomes will mature into lysosomes, in which the cargo that has not been released to the cytoplasm will be degraded (6). RNA cargo that reaches the cytoplasm can elicit its functional effect. For example, mRNA can be translated into a functional protein, such as green fluorescent protein (GFP), and the resulting fluorescence can act as a reporter of functional delivery of extracellular vesicle cargo (7). When small interfering RNA (siRNA) cargo is released into the cytoplasm, it can inhibit translation of specific transcripts, such as those encoding fluorescent proteins. In this case, disappearance of fluorescence will report on functional delivery of extracellular vesicle cargo (8). Extracellular vesicles can be tracked along this route with use of different reagents or labelling strategies (TABLE 3). 5'ppp-RNA, 5'-triphosphorylated RNA.

mRNAs. Most full-length mRNAs in extracellular vesicles are smaller than 1 kb (REF.¹¹²), but clearly, in analogy to retroviruses, longer RNA sequences could be incorporated in a condensed configuration¹¹³. mRNAs in extracellular vesicles could serve as a source of novel proteins in recipient cells. Exit from the endosome could be a bottleneck in functional mRNA delivery¹⁰¹ and may depend on the donor and/or recipient cell types. However, active translation of mRNAs transported by extracellular vesicles into recipient cells was reported early on; for example, expression of reporter proteins from mRNA transferred by extracellular vesicles between mast cells²³ and from glioblastoma to endothelial cells²⁴. In one study mRNAs transported within extracellular vesicles were translated within 1 hour after extracellular vesicle uptake during bidirectional exchange in co-cultures of glioblastoma and HEK293T cells¹¹⁴. There is also evidence that extracellular vesicle mRNA can generate active proteins, as transfer of Cre recombinase mRNA in extracellular vesicles led to recombination events in the brains of floxed reporter mice^{115,116}. Of note, this study analysed the extracellular vesicles for Cre mRNA and protein, and while the former was detected by reverse transcription-PCR, the latter was not detected by western blot or enzyme-linked immunosorbent assay. But because reverse transcription-PCR is markedly more sensitive than either of the last two protein detection techniques, the contribution of the extracellular vesicle-mediated protein transfer of Cre to this functional effect cannot be ruled out. Extracellular vesicle-mediated transfer of mRNAs between human pulmonary artery smooth muscle cells (stimulated by transforming growth factor- β) and arterial endothelial cells has also been demonstrated with a number of different mRNAs present in these extracellular vesicles,

prominently including regulators of the actin cytoskeleton and extracellular matrix remodelling¹¹⁷. Because transforming growth factor- β is a cytokine relevant for the pathology of pulmonary arterial hypertension, this extracellular vesicle-mediated communication could be involved in the disease phenotype¹¹⁷.

Importantly, as extracellular vesicles typically carry both the mRNA and its encoded protein, it is difficult to distinguish newly translated protein in recipient cells. Changing the experimental paradigm revealed that delivery of human erythropoietin mRNA to cells via lipid nanoparticles resulted in release of extracellular vesicles containing the mRNA, which was then translated in mouse cells¹¹⁸. Extracellular vesicles derived from human red blood cells have also been loaded with mRNA for Cas9 by electroporation (see more details later) and used for CRISPR editing in recipient cells¹¹⁹. These studies establish that indeed mRNA delivered by extracellular vesicles can be translated in recipient cells.

lncRNAs. The functions of lncRNAs (longer than 200 bp) are diverse, ranging from negative regulation of miRNAs by serving as miRNA 'sponges'^{120,121} to marking of mRNAs for degradation¹²² and transcriptional regulation of genes¹²³. Several studies implicate the role of extracellular vesicle-transferred lncRNAs in biological processes. One recent study documented that hypoxic cardiomyocytes release extracellular vesicles with high levels of lncRNA NEAT1, which taken up by fibroblasts induces a profibrotic gene expression programme¹²⁴. In another study, incorporation of hypoxia-inducible factor 1 α -stabilizing lncRNA into extracellular vesicles released by tumour-associated macrophages supported breast cancer cell viability¹²⁵. A future challenge will be determining the mechanism of action of these and other lncRNAs in extracellular vesicles.

tRNA fragments. Next to miRNAs, tRNA fragments are the most abundant small non-coding RNAs in extracellular vesicles. These species function in the regulation of gene expression and epigenetics, particularly in the context of immunomodulation⁴⁷ and cancer progression^{126,127}. Extracellular vesicles contain different sets of tRNA fragments that can be enriched, as shown for T cells, where 45% of these species were 1.5-fold enriched in extracellular vesicles compared with whole cells⁴⁷. Specific tRNA fragments derived from the same tRNA are sorted into extracellular vesicles, while others are not. The tRNA fragment content of extracellular vesicles appears to be dependent on the source cell state, as for T cell activation, which results in changes in the types of tRNA fragments incorporated into extracellular vesicles, with some fragments showing activation-dependent sorting into extracellular vesicles⁴⁷. Silencing of these activation-dependent tRNA fragments promoted T cell activation, suggesting that in this case, secretion of tRNA fragments is a means of removing them from the cell, thereby preventing repression of immune responses⁴⁷. Whether tRNAs and their fragments in extracellular vesicles have functional effects in recipient cells remains to be established.

Cre recombinase
A type I topoisomerase from bacteriophage P1 that catalyses cleavage and ligation of DNA at specific *loxP* recognition sites.

Table 3 | **Approaches for extracellular vesicle tracking**

Purpose of labelling	Reagent/approach	Description	Refs
Visualizing extracellular vesicles			
Membrane labelling	DiI, DiD, DiR, DiO	Lipophilic carbocyanines. Incubating donor/recipient cells with DiD labels lipid membrane via lateral diffusion	268
	CellMask	Amphipathic molecule with a lipophilic moiety and a negatively charged hydrophilic dye that anchors the probe in the membrane. Incubation of donor/recipient cells with this dye results in membrane labelling	248
	FM 4-64, FM 1-43	Lipophilic styryl compound. Incubating donor/recipient cells with this dye allows the dye to enter the outer leaflet of the surface membrane	269
	PKH26, PKH67, PKH2	Aliphatic molecules incorporated via selective partitioning into lipid membrane (of note, PKH26 can aggregate into particles, causing artefacts or false positive signals)	270
	Antibody-based tracking of extracellular vesicles	Use of Alexa Fluor-conjugated antibodies indirectly binding the extracellular vesicle proteins TSG101, CD63 and HSP70, allowing visualization of extracellular vesicles with fluorescent confocal microscopy or super-resolution microscopy	271
	Membrane labelling of extracellular vesicles with fluorescent protein	Palmitoylation signal placed in-frame of the N terminus of fluorescent protein	114
	Reporter tagging of transmembrane extracellular vesicle markers	Extracellular vesicle-associated proteins, such as flotillin 1, flotillin2, RAB5, RAB7 and CD63, can be conjugated to fluorescent proteins such as GFP and mCherry. In this way extracellular vesicles can be imaged intracellularly with time-lapse confocal imaging	272
Vesicle interior labelling (cell/extracellular vesicle-encapsulated dyes)	Carboxyfluorescein diacetate succinimidyl ester	A cell-permeable dye that labels intracellular molecules by covalently binding to intracellular lysine residues and other amine sources	273
	Calcein AM	A compound hydrolysed by intracellular esterases resulting in fluorescent signal retained within the cell	274
	CellTracker CM-DiI	Conjugates to thiol-containing peptides and proteins, retaining the fluorescent signal within the cell	275
Labelling RNA cargo	Alexa-labelled RNA	Dye that forms a stable complex with particular nucleic acids	276
	TAMRA NHS	Dye that forms a covalent amide bond with primary amine group	269
	SYTO RNASelect Green	Cell-permeant nucleic acid stains	35
Monitoring extracellular vesicle uptake			
Monitoring fusion	Quenching assays (BODIPY, NBD-rhodamine assays, octadecyl rhodamine B self-quenching)	Dyes with FRET-based self-quenching. Before fusion the probe dimer is self-quenched owing to close proximity. On fusion with a larger membrane (for example, plasma membrane or endosome) the dimer is separated and therefore no longer quenched, releasing a fluorescent signal. Therefore, detection of a fluorescent signal indicates fusion/uptake of extracellular vesicle content	277–279
	Pyrene excimer formation	Pyrene labelling lipids. Lipid probes labelled with pyrene that form dimers emit red-shifted fluorescence but when mixed with non-labelled lipids the dimer fluorescence is replaced by monomer blue-shifted fluorescence	280
Monitoring pH changes (indicating endosomal uptake)	pHluorin	pH sensitive variant of GFP	281
	pHRodo	Fluorogenic pH-sensitive dye	215
Organelle labelling	Fluorescently fused proteins	Fluorescent protein tagged to organelle-associated proteins EEA1 (early endosome), RAB5 (early/mobile endosome) and RAB7 (maturation to late endosome). Visual tracking of each compartment allows tracking of vesicles within endosomal pathway as well as visualization of escape from the endosomal pathway	276
	LysoTracker	Fluorophore linked to weak base. Labels endolysosomal compartment, allowing tracking of extracellular vesicles to the lysosome or visualization of a separation of extracellular vesicles from the endosomal pathway	282
Monitoring functional output			
Visualizing cytosolic release	β -Lactamase assay	CCF2-AM is a FRET substrate that is hydrolysed by the enzyme β -lactamase, producing a coloured product that is directly proportional to enzyme activity. Delivery of the enzyme (via a virus/extracellular vesicle) to a recipient cell expressing CCF2-AM results in a detectable fluorescent signal as a consequence of cytosolic release	283
	Split protein assay (luminescent/fluorescent)	Re-formation of separated protein fragments results in a signal. This confirms uptake of extracellular vesicle content whereby one fragment is present in extracellular vesicles and the complementary fragment is expressed in the recipient cell	284
	Evidencing mRNA translation with reporter mRNA	Exemplified by the use of reporter mRNA encoding <i>Gaussia</i> luciferase. Translation is evidenced by the signal from the reporter assay, which is prevented by treatment with the translation inhibitor cycloheximide	114

FRET, fluorescence resonance energy transfer; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl.

snoRNAs. The main function of snoRNAs is to guide chemical modification of other RNA species. snoRNAs are enriched in extracellular vesicles compared with the source cell, and after entry into recipient cells, they are shuttled to the nucleus. The snoRNA content of extracellular vesicles is influenced by the biological state of the source cell and has been shown to change in immune cells, such as dendritic cells treated with immunorepressive or immunostimulatory factors¹²⁸, and in activated macrophages¹²⁹. In the latter case, these extracellular vesicles were taken up by distant cells with an associated increase in 2'-O-methylation of RNA — a modification that the snoRNAs released by the macrophages direct on their canonical rRNA targets. Hence, extracellular vesicle-derived snoRNAs can be modulators of inflammation.

ncRNAs signalling via cellular receptors. In addition to their more specific functions, extracellular vesicle-associated ncRNAs can also produce effects via direct interactions with cytoplasmic receptors, in particular immune receptors, such as RIG-I-like receptors, which recognize pathogen-associated molecular patterns (PAMPs), and the NOD-like receptor family, which in addition to PAMPs also recognizes endogenously derived damage-associated molecular patterns (DAMPs). Most of what we know about how exRNA triggers these cytoplasmic receptors has been revealed for extracellular vesicles carrying microbial content¹³⁰. For example, *Mycobacterium tuberculosis* RNA is packed into extracellular vesicles released from infected macrophages that are subsequently taken up by other macrophages. This triggers the RIG-I RNA sensing pathway leading to production of type I interferon and accelerated maturation of bacteria-containing phagosomes by recipient macrophages, supporting increased bacterial killing¹³¹. A similar mechanism is used during latent infection with Epstein–Barr virus, whereby infected B cells release extracellular vesicles containing viral ncRNAs with the potential to stimulate RIG-I and other related PAMP receptors. These extracellular vesicles act on dendritic cells and stimulate antiviral responses¹³². There are also hints in the literature that some mammalian RNAs are able to trigger these receptor-mediated pathways. For example, cancer stromal fibroblasts increase the expression of RN7SL1 — an endogenous 5'-triphosphorylated RNA (representing a canonical target for RIG-I) — which is normally shielded by binding to its RBP. This increased expression results in unshielded RN7SL1, which is packaged into extracellular vesicles and when delivered to breast cancer cells induces RIG-I signalling and interferon-stimulated gene expression, resulting in cancer promotion¹³³ and therapy resistance¹³⁴. However, it is worth noting that in addition to these signalling responses to exRNA, any exogenous RNA may be perceived as 'foreign' by the recipient cell, leading to RIG-mediated degradation¹³⁵. In addition to proactive effects of RIG-I signalling in infection and cancer, recognition of exRNA by immune receptors can also lead to pathological inflammation and rejection of recipient cells by the activation of the immune system.

In addition to immune receptors, some exRNAs are recognized by autophagy receptors in the cytoplasm. For example, vtRNAs present in extracellular vesicles²⁷ as well as in ribonucleoprotein particles¹³⁶ can serve as important regulators of autophagy. They bind directly to the intracellular autophagy receptor sequestosome 1 (also known as p62), thereby interfering with oligomerization of the receptor and downstream clearance of protein aggregates through the autophagic pathway¹³⁷. However, the presence of vtRNA in extracellular vesicles has been challenged recently by a study showing that vtRNA was restricted to the non-vesicular exRNA in a human colon cancer cell line and was present only as a minor component in small extracellular vesicles in a human glioma line¹⁰. This discrepancy may relate to the way the vesicles were isolated in the different studies or to differences in the cells of origin (non-transformed cells versus cancer cells).

Many of the small RNAs in extracellular vesicles have the potential to influence recipient cells even without reaching the cytoplasm. These exRNAs can potentially trigger Toll-like receptors (TLRs) and thereby elicit an innate immune response¹⁰⁶. The most likely TLRs for this task are TLR3, TLR7, TLR8 and TLR9, which are intrinsically capable of sensing PAMPs and DAMPs and operate within the endosomal compartment during its maturation and acidification¹³⁸. If the lipid layer of extracellular vesicles surrounding the cargo is breached after endocytosis, the RNA cargo could be exposed inside the endosome. As such, TLRs in the endosome of the recipient cells could be triggered by recognizing this single-stranded RNA cargo. This has been demonstrated for miR-21 and miR-29a, which are secreted by lung cancer cells within extracellular vesicles, which are then taken up by surrounding immune cells in vivo and activate endosomal TLR7 and TLR8, thereby inducing a proinflammatory phenotype that supports cancer progression¹³⁹. Similarly, members of the let-7 miRNA family (let-7a and let-7e) in extracellular vesicles^{140,141} harbour a specific GU-rich motif (GUUGUGU) that binds to and activates TLR7. TLR7 activation could, in turn, impact cell function in disease-relevant contexts, including neurodegeneration¹⁴² and cancer¹⁴³.

Extracellular vesicles in viral transfer. Nucleic acids of intracellular pathogens can also be packaged into extracellular vesicles in infected cells and trigger protective responses in surrounding cells. In some cases, extracellular vesicles can also be co-opted into facilitating viral RNA transfer and, as such, aid in viral functions. Non-enveloped RNA viruses, such as leishmania RNA virus 1, hepatitis A virus and hepatitis C virus can be packaged into extracellular vesicles, serving as an invisibility cloak to shield them from host immune surveillance^{144–146}. Some viral proteins are specialized in the transmission of viral RNA via extracellular vesicle subspecies that contain elements of both the plasma membrane and the viral envelope. Intriguing reports raise the possibility that even without viral proteins, extracellular vesicles can transport functional hepatitis C virus RNA between cells in a virion-independent manner^{147,148}. Similarly, the retrovirus-derived Gag protein ARC encoded in fly⁸³ and

RIG-I-like receptors

Intracellular pattern recognition receptors that belong to the RNA box helicase family that function as cytoplasmic sensors of pathogen-associated molecular patterns elicited by viral RNA.

NOD-like receptor family

Cytoplasmic receptors expressed by innate immune and other cells. They are part of the family of intracellular pattern recognition receptors and can recognize structures associated with pathogenic microorganisms (pathogen-associated molecular patterns) or with cell damage (damage-associated molecular patterns), thereby activating the innate immune response and inflammation.

Type I interferon

A large subgroup of cytokines that help to regulate the activity of the immune system. They act to degrade viral DNA and proteins in infected cells and provide protection to surrounding cells through interaction with and signalling from interferon receptors.

Toll-like receptors

(TLRs). A class of transmembrane proteins involved in the defence of the organism through innate immunity. They are mainly expressed on the membrane of sentinel cells, such as macrophages and dendritic cells, and recognize certain typical structures of pathogens and microorganisms.

mammalian¹⁴⁹ genomes can form and release enveloped capsids that contain their own mRNA (about 1 kb). The *trans*-synaptic delivery of these extracellular vesicles between neurons and expression of the encoded ARC RNA in postsynaptic neurons were found to be critical in modulating synaptic plasticity.

Modulating RNA cargo and its delivery

Incorporating selected RNA cargo into extracellular vesicles with the potential for targeting to specific cell types *in vivo* has immense therapeutic potential. One important issue in functional extracellular vesicle transfer is the type of cells the vesicles are obtained from. Depending on the clinical use, cell sources include mesenchymal stem cells, which can be isolated from a number of different tissues and are intrinsically regenerative and non-inflammatory^{150,151}; red blood cells of type O donors, which are easily loaded with cargo¹⁵²; and dendritic cells, which can present antigens and activate T lymphocytes and thus be used for vaccination^{153,154}. In the last context, extracellular vesicles from tumour cells could be used to elicit antitumour immune responses via presentation of tumour-associated antigens. However, there is concern that vesicles isolated from immortalized cells or tumour cells may carry oncogenic factors and contribute to cancer¹⁵⁵.

As mentioned earlier, extracellular vesicles have many similarities to enveloped viruses, which can serve as prototypes to understand the potential for these vesicles to act as vectors for delivery of functional RNAs. Enveloped coronaviruses, for example, have a size similar to that of extracellular vesicles (125 nm) and contain single-stranded RNA of up to 30 kb. This is the largest reported genome size of any RNA virus and may serve as a benchmark for the maximum RNA capacity of these vesicles when packaged in a condensed form. Nevertheless, in addition to RNA, extracellular vesicles contain many other types of cargo, which might have to be displaced to allow full RNA-loading capacity. Different approaches are being explored for loading RNA into extracellular vesicles, targeting strategies and production considerations.

Packaging selected RNA into extracellular vesicles. Loading of the desired cargo for transfer via extracellular vesicles can be achieved either endogenously, by modulation of the donor cell to favour inclusion of the selected cargo into extracellular vesicles, or exogenously, in which case the cargo is loaded into isolated extracellular vesicles *in vitro*.

In endogenous loading, overexpression of an RNA in the source cell through DNA transfection or lentiviral transduction usually results in enrichment of that RNA, as well as any encoded protein, in the extracellular vesicles generated by those cells^{156,157}. The yield of RNA-loaded extracellular vesicles can be further increased by mechanical extrusion of vesicles through a micrometre-pore-sized filter using cells that express high levels of the desired mRNA¹⁵⁸. RNA enrichment can also be achieved by having the donor cells express a protein that both interacts with the plasma membrane (for example, via different lipid anchors)¹⁵⁹ or

a viral envelope protein (such as vesicular stomatitis virus glycoprotein)¹⁶⁰ and has an RNA-binding motif, typically of viral origin (for example, the RNA-binding peptide from the MS2 bacteriophage coat protein)¹⁶⁰. In concert, the cargo RNA is equipped with specific sequences recognized by the RNA-binding motif in the protein, allowing targeting of the RNA of interest to the plasma membrane and facilitating its release through budding from the plasma membrane, mimicking retrovirus-like particle release¹⁵⁹ (FIG. 2). It is also possible to target RNAs to MVBs, by expressing protein fusions that couple an MVB-enriched protein (such as lysosome-associated membrane protein 2, tetraspanin or CD63) with an RNA-binding motif^{161,161}. One approach for selective enrichment in extracellular vesicles took advantage of a specific subpopulation of microvesicles, referred to as 'arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles'. In this approach, ARRDC1 and RNA cargo were engineered to interact — via including the transactivator of transcription (Tat) peptide from HIV in ARRDC1 and its RNA binding-motif, transactivating response (TAR) element, in the RNA — allowing efficient generation of RNA cargo-loaded extracellular vesicles. This strategy was successful in transfer of p53 mRNA, which was translated in recipient cells on uptake (including *in vivo* delivery into mice)¹⁶². Beyond the use of viral components, other approaches have included incorporation of 'zipcode' sequences, typically in the 3' untranslated region of the cargo RNA, which can be recognized by specific RBPs associated with membrane-targeted or MVB-targeted proteins (see, for example, REFS^{85,161}). Taking advantage of the lipid biocomposition of extracellular vesicles can also help in loading RNA-RBP complexes into them, by engineering RBPs to bind to extracellular vesicle membranes more efficiently via addition of specific peptides. The different lipids in extracellular vesicles make possible binding with curvature-sensing and lipid composition-specific affinity peptides. Annexin A5 is a well-known example that binds to negative curvature-specific lipid phosphatidylserine on apoptotic bodies and extracellular vesicles, while the effective domain (residues 151–175) of myristoylated alanine-rich C-kinase substrate (MARCKS) protein has an affinity for positive-curvature-associated lipid, phosphatidylinositol 4,5-bisphosphate that is present on plasma membrane-derived vesicles¹⁶³. Some other proteins, such as bradykinin, can also harbour peptides that bind highly curved membranes¹⁶⁴. Other options include incorporation of binding domains of proteins with affinity for intracellular membranes, such as phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 3,4,5-trisphosphate¹⁵⁹.

Common methods to load RNA into isolated extracellular vesicles exogenously include electroporation of siRNAs (see, for example, REFS^{119,165–169}), antisense oligonucleotides¹¹⁹ and mRNAs¹¹⁹, as well as use of sonication¹⁷⁰ and Lipofectamine¹⁶⁹. One of the most promising approaches uses chemically stabilized siRNAs bearing a lipophilic moiety, such as cholesterol, which results in high incorporation onto and through the lipid membrane of the extracellular vesicles with documented

Lysosome-associated membrane protein 2B

A particular isoform of LAMP2 — a protein that associates with the membrane of multivesicular bodies and lysosomes.

Tetraspanin

A family of membrane proteins consisting of four transmembrane domains, with intracellular N and C terminals and two extracellular domains. Tetraspanins are present in the membranes of extracellular vesicles.

'Zipcode' sequences

Sequences typically found in the 3' untranslated regions of mRNA transcripts that can be recognized by specific RNA-binding proteins.

Lipofectamine

A transfection reagent, composed of a cationic tail and a lipid region. Lipofectamine binds exogenous DNA and RNA, creating liposomes that, once in contact with the plasma membrane of the cells, merge with it and facilitate entry of nucleic acids into the cytoplasm.

functional knockdown of target mRNA in vivo^{171,172}. Still, it is important to keep in mind that isolated extracellular vesicles are inherently already 'full' of RNA and proteins, and it is not clear how much more additional cargo can be effectively loaded into them. Nevertheless, the lipid membrane may be extendable, and stabilized siRNAs can be loaded on both the inside and the outside, thereby supporting these in vitro loading strategies. In considering different modes of loading of RNA into isolated extracellular vesicles, one should also keep in mind the integrity of the vesicles and potential antigenic/toxic components included in the loading process, which may limit successive administrations of exogenously loaded vesicles.

Strategies to control extracellular vesicle yield and stability.

In addition to cargo loading, to ensure efficient cell–cell communication (especially in a therapeutic setting), it is key to ensure high yields of high-quality extracellular vesicle production¹⁷³. Generally, obtaining large amounts of pure extracellular vesicle preparations necessitates copious starting material, and enhancing the production of extracellular vesicles is a current focus of many researchers and biotechnology companies. Increasing extracellular vesicle biogenesis in the donor cell is one strategy to increase the overall availability of extracellular vesicle RNA. One possibility is to overexpress regulatory proteins involved in extracellular vesicle biogenesis. A screen with a readout for the numbers of extracellular vesicles released identified factors in the donor cell that promoted exosome biogenesis, including STEAP3 (involved in exosome biogenesis), syndecan 4 (MVB formation) and an increase in the citric acid cycle to boost production of RNA cargo-loaded exosomes¹⁶¹. In another study, overexpression of the actin-bundling protein cortactin to increase MVB docking at the plasma membrane (see FIG. 2) proved efficient in increasing exosome release⁵⁶. Treatment of source cells with different compounds, such as bafilomycin A1 (a V-ATPase inhibitor¹⁷⁴) or heparanase¹⁷⁵, which regulates secretion, composition and function of tumour cell-derived exosomes, also increased production of extracellular vesicles. Extracellular vesicle secretion was also increased by elevated levels of intracellular Ca²⁺ in response to treatment with monensin, a Na⁺/H⁺ exchanger¹⁷⁶. Natural compounds can influence extracellular vesicle release; for example, microglia exposed to serotonin exhibited increased fusion of MVBs with the plasma membrane, via increase in cytosolic Ca²⁺ concentration¹⁷⁷. Radiation, hypoxia, oxidative stress and low pH can all upregulate extracellular vesicle production^{178–181}. Vesicular stomatitis virus glycoprotein also promotes extracellular vesicle release^{182,183}. Finally, the yield of isolated extracellular vesicles can be increased by in vitro fusion with synthetic lipids, followed by extrusion through small pores (100–400 nm in diameter¹⁸⁴). These vesicles could then be loaded with cargo (siRNA) via electroporation and were successfully taken up by recipient cells in culture at a rate that was more than tenfold higher than that of native vesicles¹⁸⁴.

Although increased production of extracellular vesicles is generally desired for therapeutic purposes,

inhibition of extracellular vesicle release from tumour cells has been proposed as a therapeutic strategy to curtail advancement of cancer. A number of compounds have been identified that reduce secretion of extracellular vesicles. For example, the antihistamine drug ketotifen¹⁸⁵ and the antibiotic sulfisoxazole¹⁸⁶ curb extracellular vesicle release in multiple cancer cell lines. Screens for modulators of extracellular vesicle secretion have yielded various chemical compounds that both increase secretion (for example, sitafloxacin, chlorindole and fenoterol) and reduce secretion (for example, manumycin A, tipifarnib and neticonazole)¹⁸⁷. In a different approach, breast cancer-derived exosomes were removed from the circulation through extracorporeal haemofiltration in an attempt to decrease growth of breast cancer in patients¹⁸⁸.

Despite the progress in regulating extracellular vesicle production and loading of cargo, many of the strategies in place so far use treatments that may alter cell phenotypes and extracellular vesicle functionality and/or have a toxic or immunogenic response in vivo. Further work will be needed to expand and evaluate these methods for research and clinical use.

Targeting extracellular vesicles to specific cell types and promoting uptake.

Similarly to viral gene therapy, a major challenge in using extracellular vesicles for therapeutic purposes is achieving delivery to diseased cells in living organisms. Extracellular vesicles administered in vivo suffer from rapid clearance mostly by uptake into cells in the liver, kidneys, lungs and spleen^{189–191} with different routes of administration, including intravascular, intraperitoneal and subcutaneous, altering the distribution only to a minor extent¹⁹¹. Overall, goals include increased targeting of therapeutic extracellular vesicles to specific cell types, facilitation of their translocation through different biological barriers and extending the half-life of vesicles in extracellular spaces in vivo. Some increase in stability in vivo has been achieved by coating extracellular vesicles with the synthetic polymer polyethylene glycol¹⁹² or displaying CD47 on their surface, which decreases phagocytosis by macrophages¹⁶⁸.

More selective targeting to tissues/cells of interest could be achieved by the incorporation of specific ligands on the surface of extracellular vesicles, including proteins/peptides, antibodies, lipid-associated entities and RNA aptamers¹⁹³ that will be recognized by the acceptor cell (for a review, see REF.⁹⁰). In an early attempt to route extracellular vesicles to the brain after intravenous delivery, a peptide from rabies virus glycoprotein that targets acetylcholine receptor-expressing cells in the brain was fused to lysosome-associated membrane protein 2B (which associates with MVBs)¹⁶⁵, but its effectiveness differs in different studies. Extracellular vesicles have also been engineered to display a nanobody to the epidermal growth factor receptor on their surface to target tumour cells expressing this receptor¹⁹⁴. Tissue-targeting ligands can also be linked to the phosphatidylserine-binding domain of lactadherin, which is exposed on the surface of extracellular vesicles¹⁹⁵. In yet another approach, a single-chain variable fragment of an anti-HIV monoclonal antibody was incorporated into an expression

V-ATPase

Vacuolar-type H-ATPase. A transmembrane protein found mainly in intracellular organelles and the plasma membrane. It catalyses the hydrolysis of ATP, allowing transport of solutes and acidification of organelles.

Heparanase

Heparanases are a group of enzymes that act both at the cell surface and within the extracellular matrix to degrade polymeric heparan sulfate molecules into shorter chain length oligosaccharides.

Gene therapy

A variety of methods resulting in alterations in the genome and/or transcriptome of cells in order to correct deficiency or dominant negative effects caused by genetic mutations or infections.

RNA aptamers

Small oligonucleotides that bind to target molecules with high affinity and specificity through their 3D structures.

vector designed to target recombinant proteins to the surface of mammalian cells to deliver apoptosis-inducing miR-143 (or the drug curcumin) to kill HIV-infected cells¹⁹⁶. Finally, a high-throughput screening method (phage display) was used to identify a peptide, CP05, that binds to an extracellular domain of tetraspanin CD63 (REF.¹⁹⁷). By linking muscle-targeting peptides and antisense oligonucleotides to CP05, efficient delivery of these antisense RNAs to skeletal muscle in a mouse model of Duchenne muscular dystrophy was achieved¹⁹⁷. As an alternative strategy to incorporation of targeting ligands directly in the extracellular vesicle membrane, it is also possible to incorporate artificial lipids into extracellular vesicle membranes, where they can aid in coupling of tissue-targeting antibodies or homing peptides with the extracellular vesicle surface and thus influence the biodistribution of therapeutic extracellular vesicles¹⁹⁸.

Besides modification with proteins/peptides, there is growing interest in functionalizing extracellular vesicles with RNA aptamers to control their targeting. In this regard, a bacteriophage Φ 29 RNA, which has a three-way junction allowing its self-association, has been engineered to incorporate a membrane-targeting cholesterol moiety, a prostate-specific membrane antigen or RNA aptamer, and an epidermal growth factor receptor RNA aptamer¹⁹⁹. This RNA trimer composite incorporated itself spontaneously into membranes of extracellular vesicles *ex vivo*. These decorated extracellular vesicles could then be loaded with RNA cargo (such as anticancer siRNAs) to inhibit tumour growth in mouse models. Cancer cell-targeting aptamers were also successfully conjugated to a hydrophobic diacyl lipid tail and incorporated in extracellular vesicle membranes following their loading with anticancer drugs²⁰⁰. Extracellular vesicle functionalization has also been performed chemically (via reaction between aptamer-conjugated aldehyde and the amino group of membrane proteins incorporated into extracellular vesicles), whereby extracellular vesicles from bone marrow stromal cells were equipped with a bone marrow-specific RNA aptamer²⁰¹. These functionalized extracellular vesicles were then efficiently targeted to the bone marrow after intravenous injection in mice and promoted bone regeneration. By contrast, non-functionalized extracellular vesicles primarily accumulated in the liver and lungs and did not have beneficial effects on bone regeneration²⁰¹.

Overall, a wide variety of specific targeting strategies are being explored for extracellular vesicle delivery. One major caveat of these strategies, however, is that such ligands in the form of non-protected oligonucleotides and peptides/proteins exposed on the surface of the extracellular vesicle may be digested off in biofluids by RNases and proteases, respectively, thereby preventing targeting^{202,203}.

More global approaches for directing tissue accumulation of extracellular vesicles following intravenous administration include chemical modification of their surfaces. For example, treatment with neuraminidase to neutralize the negative charges on extracellular vesicles contributed by polysaccharides and glycosylated phospholipids reduced extracellular vesicle targeting

to the liver, with a larger proportion of vesicles taken up by other organs, such as the brain, after intravenous injection in mice²⁰⁴. Engineered glycans can also be displayed on the surface of extracellular vesicles, as these moieties appear to be important for extracellular vesicle recognition and uptake by recipient cells^{205,206}. Increased cellular uptake in general can be achieved by the addition of cationic lipids (for example, in the form of Lipofectamine) to the surface of extracellular vesicles²⁰⁷, by increasing macropinocytosis with an arginine-rich cell-penetrating peptide exposed on the surface of the extracellular vesicles²⁰⁸ or by inducing receptor clustering on recipient cells²⁰⁹.

Increasing cytosolic delivery of the RNA cargo. Cytosolic delivery of extracellular vesicle content can potentially be improved by enhancing the fusogenic properties of extracellular vesicle membranes, which should result in the direct deposition of cargo RNA into the cytoplasm of the cell. Direct fusion of the extracellular vesicles with the plasma membrane can be achieved by incorporating fusion proteins, such as syncytin 1, syncytin 2 and epithelial fusion failure 1 protein²¹⁰, into their membranes. Furthermore, the gap junction protein connexin 43 is present in the extracellular vesicle membrane and can create a channel in the plasma membrane that allows passage of oligonucleotides into the cytosol²¹¹. On the basis of the similarity between extracellular vesicles and viral particles, incorporation of viral proteins, such as vesicular stomatitis virus glycoprotein¹⁸³, into extracellular vesicles is an appealing strategy to target extracellular vesicles to specific cell types and organs while also improving functional delivery of RNA, albeit at the risk of introducing antigenic proteins^{113,212}. For escape from the endosomal pathway, ideas can be sought from bacteria and viruses. Some enveloped and non-enveloped viruses (for example, adeno-associated virus and influenza virus) are taken up by endocytosis and through pH-sensitive fusion proteins use the acidic pH of the endosomal compartment to promote membrane fusion between the endosomal and vesicular membranes^{213–215}. Many species of pathogenic bacteria produce pore-forming toxins to escape from the degradative route following their uptake into host cell endosomes^{216–218}. Such proteins could be engineered into extracellular vesicle membranes. Addition of compounds such as chloroquine that block acidification of the endosomal compartment and release of viruses paradoxically enhanced extravascular delivery in one of the studies, possibly through a protein sponge effect leading to swelling and bursting of the endosomes²¹⁹. Other compound found to increase endosomal escape was small molecule UNC10217832A²¹⁹.

For liposome-based delivery, increased efficiency of endosomal escape was achieved by inclusion of an amine-rich polymer (polyethylene imine)²²⁰. While this may not be directly applicable to extracellular vesicles, it does highlight the existence of compounds that can specifically increase rates of endosomal escape and might improve intercellular delivery of extracellular vesicle content, with the possibility of combining liposome components with extracellular vesicles²²¹.

Potential clinical applications

Extracellular vesicles show promise for clinical applications both as biomarkers and as therapeutic delivery vehicles. Here we focus on uses of their RNA cargo, but proteins, lipids and glycans can all be informative as biomarkers that carry the information about their cellular source, whereby their make-up informs on the physiological state, with changes in composition often associated with a disease state. With their use as biomarkers, the main issue is detecting the subset of extracellular vesicles from a particular tissue in a sea of vesicles from other cell sources in biofluids. For therapy, extracellular vesicles can be used to deliver not only RNA but also proteins and drugs and combinations thereof. Issues remain concerning best practices for isolating large numbers of clinical grade quality vesicles, loading them and directing their biodistribution to target tissues. In most scenarios they would be used to promote delivery of therapeutic cargo — be it functional RNA or protein — to the diseased tissue or tumour and would need to be readministered repeatedly throughout the disease state. Nevertheless, extracellular carriers can also serve as carriers of CRISPR technology to induce pathological error correction, and in this case they might be effective in a single dose.

Use of extracellular vesicle RNA cargo in diagnostics.

RNAs in extracellular vesicles are in essence a snapshot of the content and state of the cells that secrete them and are therefore very useful for biomarker discovery⁶¹. Extracellular vesicles are found in a wide variety of biofluids, including serum/plasma, urine, saliva, cerebrospinal fluid and breast milk^{222,223}. An advantage of using extracellular vesicle-derived RNAs as biomarkers is that they are protected from degradation by RNases and thus are quite stable, and that the vesicles display on their surface the antigenic markers of the cells from which they were derived, allowing enrichment of vesicles from a particular tissue source (for example, using antibody capture for the enrichment of tumour-derived vesicles in serum²²⁴). Many new sensitive technologies, such as Droplet Digital reverse transcription-PCR are being used to enhance detection of specific RNA species in extracellular vesicles²²⁵.

RNAs can serve as biomarkers based on the mutations, splice variants and fused mRNAs as well as alterations in levels of specific RNAs associated with various disease states. The first example using extracellular vesicles in biofluids was the detection of a tumour-specific mRNA: mutant epidermal growth factor receptor in serum of patients with glioblastoma²⁴. In other examples, elevated levels of specific miRNAs have been found in cerebrospinal fluid from patients with glioblastoma²²⁶, patients with Alzheimer disease and Parkinson disease²²⁷, and patients with breast cancer²²⁸. Changes in levels of mRNA splice variants are detected in urine from patients with myotonic dystrophy²²⁹. lncRNA profiles in blood samples from patients with type 2 diabetes correlate with heart functions²³⁰. This biomarker application of extracellular vesicles has already been successfully translated into the clinical setting. Currently, mutated mRNAs in extracellular vesicles and other carriers in plasma are being used as biomarkers for the diagnosis of haematological

disorders²³¹ (for example, NeoLAB liquid biopsy from NeoGenomics Laboratories) and for prognostic assessment of prostate, lung and other solid tumours^{232,233} (for example, ExoDx prostate test from Bio-Techne).

Therapeutic RNA delivery via extracellular vesicles.

There is tremendous interest in using extracellular vesicles as vehicles to deliver RNA therapeutics. Extracellular vesicles as therapeutic carriers have several advantages: they can be obtained from a variety of sources, including the patient's own extracellular vesicles (in which case they would be immune neutral); they protect the cargo within a lipid bilayer; they provide a membrane platform for displaying ligands; and they can carry not only RNA but also other therapeutic agents at the same time. At the present time, these applications are still limited by the inherent heterogeneity of extracellular vesicles and the inability to achieve a fully characterized pure extracellular vesicle population. Extracellular vesicle preparations being used for preclinical trials are more appropriately referred to as 'secretomes' as they contain a mixture of extracellular elements^{234,235}. Despite these limitations, therapeutic RNA delivery via extracellular vesicles has already entered clinical investigations (see REF.¹⁵⁰ for a review). Preclinical studies have shown that unmodified mesenchymal stem cell-derived extracellular vesicles are distributed broadly after intravenous injection^{236,237} and can ameliorate the effects of various diseases in the liver, brain, heart, kidneys, lungs, skeletal muscle and immune system²³⁸. There are now also strategies in place to obtain extracellular vesicles in considerable amounts from various sources, such as commonly used cells that can be expanded on a large scale (for example, mesenchymal stem cells^{172,239}, red blood cells²⁴⁰ and HEK293 cells²⁴¹), biofluids that are readily available in large volumes (for example, bovine milk²⁴² and human plasma¹⁶⁷) and even plant materials (for example, grapefruit²⁴³ and ginger¹⁶⁷). Vesicles and therapeutic agents generated from plants and other food items have the advantage that they fall into the category of natural substances and as such are not under the strict guidelines of the US Food and Drug Administration for clinical trials²⁴⁴. Furthermore, as discussed earlier, extracellular vesicles can be further modified to be loaded with cargo, generating 'designer' vesicles with increased production, cargo loading and/or targeting for improved therapeutic outcome.

Five basic variations on the theme of using extracellular vesicles as therapeutic vehicles are being tested preclinically or in clinical trials: implantation of donor cells, such as mesenchymal stem cells, which release extracellular vesicles on site; injection of purified, unmodified extracellular vesicles from cultured donor cells; injection of purified extracellular vesicles that have been modified for targeting or loading of cargo in vitro; injection of extracellular vesicles enriched in cargo of interest and/or with enhanced targeting/uptake properties from genetically modified cells; and implantation of genetically modified cells that release modified extracellular vesicles in vivo (FIG. 4). A few examples of these strategies taken from a large literature are provided here. First, mesenchymal stem cell-derived extracellular vesicles with their intrinsic composition of RNAs and

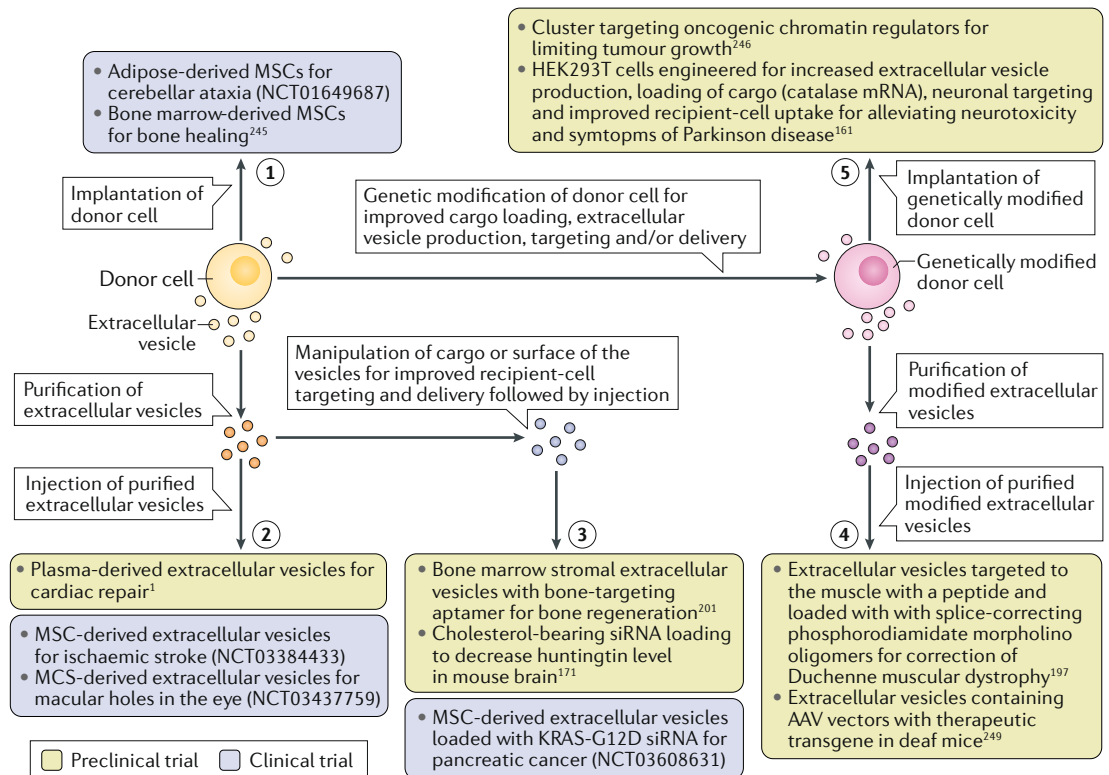


Fig. 4 | Different strategies for using extracellular vesicles for therapeutic applications. Examples of therapeutic applications of extracellular vesicles that have resulted in either preclinical assessment or clinical trials (references to primary research or clinical trials are given). Generally, there are five strategies that can be used: implantation of donor cells, such as mesenchymal stem cells (MSCs), which release extracellular vesicles on-site (1); injection of purified, unmodified extracellular vesicles from cultured donor cells (2); injection of purified extracellular vesicles that have been modified for targeting or loading of cargo in vitro (3); injection of extracellular vesicles enriched in the cargo of interest and/or with enhanced targeting/uptake properties from genetically modified cells (4); and implantation of genetically modified cells that release modified extracellular vesicles in vivo (5). AAV, adeno-associated virus; siRNA, small interfering RNA.

proteins are being evaluated for treatment of cerebellar ataxia ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01649687) identifier NCT01649687), bone repair²⁴⁵ and regeneration of macular holes in the eye (NCT03437759), although the role of specific RNAs in therapeutic efficacy has not yet been defined. Extensive effort is directed at loading isolated extracellular vesicles and adding targeting moieties to their surface to improve cargo delivery. For example, mesenchymal stem cell-derived extracellular vesicles transfected with miR-124 — an miRNA most abundantly expressed in the central nervous system and negatively associated with neural progenitor differentiation — are being tested as treatment for ischaemic stroke (NCT03384433), and mesenchymal stem cell-derived extracellular vesicles loaded with an miRNA against mutant KRAS are being tested as a therapy for pancreatic cancer (NCT03608631). In another example, loading of extracellular vesicles with cholesterol-conjugated siRNA to huntingtin mRNA has been shown to be effective in lowering levels of the protein in a mouse model of Huntington disease¹⁷¹. As discussed earlier, including a bone-targeting aptamer on the surface of extracellular vesicles derived from bone marrow stromal cells is an efficient strategy to therapeutically target the bone marrow in a mouse model of osteoporosis²⁰¹. It is also possible to harvest extracellular vesicles

from genetically modified producer cells presenting targeting peptides, load them with desired cargo and administer them in vivo. This strategy alleviated pathology in a mouse model of Duchenne muscular dystrophy, where extracellular vesicles were designed to target muscle and loaded with splice-correcting antisense nucleotides targeting dystrophin (the alteration of which causes pathology of Duchenne muscular dystrophy)¹⁹⁷. On the front of using entire cells rather than purified extracellular vesicle administration, preclinical studies have explored genetically modified glioma cells expressing high levels of a cluster of miRNAs (which are incorporated into extracellular vesicles) to suppress growth of non-modified glioma cells in mouse brain²⁴⁶. In another study, increased yields of extracellular vesicles from HEK293T cells loaded with mRNA for the antioxidant enzyme catalase with ligands for neuronal targeting and endosomal escape were effective in combatting neurotoxicity and Parkinson disease-associated neurodegeneration in mice after subcutaneous implantation¹⁶¹.

Potential advantages associated with use of extracellular vesicles compared with other RNA delivery strategies, such as synthetic lipid nanoparticles or naked modified RNA molecules, include lower immunogenicity and toxicity^{241,247}, better ability to cross cell and tissue

Major histocompatibility complexes

A group of genes encoding plasma membrane proteins that facilitate recognition of foreign peptides by the adaptive immune system.

barriers^{21,110} and the potential to achieve more efficient delivery into cells²⁴⁸, while maintaining the possibility of incorporating targeting molecules and of loading both endogenous and synthetic RNA cargo. There is also potential for the use of extracellular vesicles in combinatorial therapeutic approaches. An emerging strategy of combining extracellular vesicle-mediated delivery with gene therapy showed improved therapy for hearing loss when adeno-associated viruses harbouring therapeutic transgenes were packaged into extracellular vesicles in a mouse model of hereditary deafness²⁴⁹. Extracellular vesicles²⁵⁰ and exosome–liposome hybrids²⁵¹ can also be used for delivery of Cas9 and potentially the guide RNA as well for CRISPR gene editing.

Some of the first clinical trials using extracellular vesicles involved their use as vaccines, specifically those targeting tumours. In this case, dendritic-cell-derived extracellular vesicles harbouring on their surface major histocompatibility complexes, which before injection were pulsed with tumour-specific peptides, thereby providing a platform for efficient delivery of immunogenic tumour antigens, were injected into patients (see, for example, REF.²⁵²). In further development of extracellular vesicles as vaccines, it is possible that extracellular vesicle delivery of mRNAs for pathogenic proteins into dendritic cells (see, for example, REF.²⁵³) might potentiate vaccination without the need to generate and inactivate pathogenic viruses or bacteria. Similarly, dendritic cell-derived extracellular vesicles can be loaded with total tumour RNA or lysate, providing an abundant source of tumour-specific antigens²⁵⁴. In another avenue of using extracellular vesicles in antitumour vaccination, it was shown that tumour-derived extracellular vesicles can promote the antitumorigenic responses in dendritic cells. In this case extracellular vesicles from the tumour can be used directly or could be used for preloading dendritic cells before their implantation into the patient (as dendritic cell-based antitumour vaccines), with the latter showing so far greater therapeutic potential.

Given the promising results from early studies of extracellular vesicles as therapeutics, substantial investment is being made in this area, both from funding sources such as the US National Institutes of Health (with 302 currently funded grants) and from venture capital firms (at least ten [biotechnology companies](#)).

Conclusions and perspective

While there is consensus that RNAs in extracellular vesicles mediate communication among cells, and there are demonstrated examples of specific RNAs carried

by extracellular vesicles from one cell type mediating defined phenotypic effects on target cells, many questions remain to be resolved before we will be able to fully decipher this complex language. There are a variety of types of extracellular vesicles that differ in size and protein, lipid and RNA cargo. So far, attempts to characterize unique extracellular vesicle subclasses have proved challenging, in large part owing to technical and biological limitations and challenges involved in separation and molecular analysis of these nanoscale structures. Moreover, the RNA content of extracellular vesicles is quite complex, mostly comprising small non-coding RNAs, the function of most of which are just beginning to be elucidated. There is growing evidence that different extracellular vesicle subclasses carry different RNA cargo, but a comprehensive cataloguing of this cargo awaits the development of efficient methods to identify and separate extracellular vesicle subclasses. It is possible that some extracellular vesicles have a higher content of functional RNAs and others serve more as ‘rubbish bags’ for the source cell (still, jettisoning of certain RNAs can have functional consequences for both source cells and recipient cells). In terms of extracellular vesicle-mediated signalling, the modes by which extracellular vesicle subclasses are ‘addressed’ to different recipient cell types await delineation. Although we know miRNAs and mRNAs in extracellular vesicles can be transferred to and be active in recipient cells, it remains to be determined what percentage of the RNA cargo performs functions and what percentage is lost to degradation (or is simply not functional in the recipient cell). A key issue here is the endosomal escape routes of RNA in extracellular vesicles, as this step appears to be the major bottleneck in efficient functional delivery of cargo. Given the apparent roles of extracellular vesicles in physiology and disease, understanding these issues is critical to providing insights into pathogenic mechanisms and potential therapeutic interventions. In this Review, we have provided a platform of the current state of knowledge of RNA transported in extracellular vesicles on which the field can build another level of understanding. In parallel, there is a burgeoning field of nanoparticles that can efficiently carry mRNA and siRNA to cells and escape the endosome (see, for example, REFS^{255,256}). It will be important for the extracellular vesicle and nanoparticle/liposome fields to engage in dialogue and combine the best properties of both for therapeutic applications²⁵⁷.

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Competing interests

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