INNOVATION

Extracellular vesicles: biology and emerging therapeutic opportunities

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Abstract | Within the past decade, extracellular vesicles have emerged as important mediators of intercellular communication, being involved in the transmission of biological signals between cells in both prokaryotes and higher eukaryotes to regulate a diverse range of biological processes. In addition, pathophysiological roles for extracellular vesicles are beginning to be recognized in diseases including cancer, infectious diseases and neurodegenerative disorders, highlighting potential novel targets for therapeutic intervention. Moreover, both unmodified and engineered extracellular vesicles are likely to have applications in macromolecular drug delivery. Here, we review recent progress in understanding extracellular vesicle biology and the role of extracellular vesicles in disease, discuss emerging therapeutic opportunities and consider the associated challenges.

Cell to cell communication is pivotal for all multicellular organisms. Cells exchange information through the secretion of soluble factors or by direct interaction. In addition, most eukaryotic cells release membrane-derived vesicles that can have an impact on both neighbouring and distant cells[1]. Such extracellular vesicles were initially described nearly 30 years ago when two independent groups observed that multivesicular bodies in reticulocytes released such vesicles into the extracellular space[2,3]. Since then, extracellular vesicles have been purified from nearly all mammalian cell types, including stem cells[4–8], primary cells of the immune and nervous systems[9–13] as well as numerous cancer cell lines[14–16] (reviewed in REF. 17). Importantly, the secretion of extracellular vesicles is not restricted to mammalian cells but has also been identified in lower eukaryotes and prokaryotes[18–20].

Perhaps unsurprisingly, extracellular vesicles were initially regarded as membrane debris with no real biological significance. However, in 1996, Raposo *et al.*[21] showed that extracellular vesicles could stimulate adaptive immune responses. Since then, the importance of extracellular vesicles in intercellular communication — via the transfer of proteins, lipids and nucleic acids — has been affirmed in numerous studies[1,17,22–24]. Extracellular vesicles have been isolated from most bodily fluids and it is increasingly evident that they have a key role not only in the regulation of normal physiological processes, such as stem cell maintenance[4], tissue repair[25], immune surveillance[21] and blood coagulation[26], but also in the pathology underlying several diseases[1].

Extracellular vesicles have been tightly linked to tumorigenesis[5,27], the spread of viruses and pathogenic agents such as HIV-1 (REF. 28), amyloid-β-derived peptides[29] and α-synuclein[30] (which are pathologically linked to Alzheimer's disease and Parkinson's disease, respectively), as well as the spread of the abnormal pathogenic cell surface prion protein PrP^C (REF. 31). Extracellular vesicles and their components therefore represent a novel class of therapeutic targets. Moreover, recent data indicate that extracellular vesicles may also be exploited directly as potential therapeutic agents for tissue regeneration and immune response modulation. For example, extracellular vesicles from mesenchymal stem cells (MSCs) have been used to stimulate tissue repair following myocardial infarction[6–8], and extracellular vesicles derived from tumour antigen-pulsed dendritic cells (DCs) have been exploited for cancer immunotherapy[32].

Finally, as extracellular vesicles can transport nucleic acids as part of normal cell to cell communication[33–35], these vesicles also have potential as drug delivery vehicles[34–37]. Here, we discuss the role of extracellular vesicles in normal biological processes as well as aberrant pathological processes and focus on how extracellular vesicles can be targeted or directly exploited for therapeutic intervention.

Classification and biogenesis

Extracellular vesicles are classified based on their cellular origin and/or biological function or based on their biogenesis (BOX 1). As determined by their biogenesis, the three main classes of extracellular vesicles are exosomes, microvesicles and apoptotic bodies. Here, we focus on the first two classes of extracellular vesicles. The common denominator is that they are all cell-derived vesicles that are enclosed by a lipid bilayer, ranging from 30 nm to 2,000 nm in diameter depending on their origin. In contrast to microvesicles, which are generated by budding from the plasma membrane, exosomes are derived from the endolysosomal pathway (FIG. 1). Therefore, although they are heavily enriched in phosphatidylserine, the membrane composition of microvesicles reflects that of the parent cell more closely than does the membrane composition of exosomes. Both extracellular vesicle types contain cytoplasmic proteins, certain lipid raft-interacting proteins and RNAs but, owing to their highly regulated biogenesis, exosomes typically accommodate some additional defined components[1,23] (BOX 1).

Several mechanisms have recently been identified to regulate exosome biogenesis, thus facilitating protein and RNA cargo sorting to generate exosomes with a precise biochemical composition[22,38–40] (FIG. 1). Despite these recent advances, the terms 'exosome' and 'microvesicle' have been used interchangeably in many published studies,

Box 1 | **Classification of extracellular vesicles**

Extracellular vesicle classification can be based on their cellular origin or biological function; alternatively, extracellular vesicles can be categorized on the basis of their biogenesis pathways.

Cellular origin and biological function

- Ectosomes: vesicles secreted by neutrophils or monocytes
- Microparticles: vesicles shed from platelets in blood or endothelial cells
- Tolerosomes: vesicles purified from serum of antigen-fed mice
- Prostatosomes: vesicles extracted from seminal fluid
- Cardiosomes: vesicles secreted by cardiomyocytes
- Vexosomes: vesicles linked with adeno-associated virus vectors

Biogenesis

Distinct biogenesis pathways lead to different types of extracellular vesicles, as described in the table below. However, extracellular vesicle markers are not exclusively specific and the same markers can also be present in other types of vesicles. Owing to the overlap, the markers presented here only describe which proteins are enriched in a particular vesicle type compared with other vesicle types.

Vesicle types	Characteristics			
	Origin	Size	Markers	Contents
Exosomes	Endolysosomal pathway; intraluminal budding of multivesicular bodies and fusion of multivesicular body with cell membrane	40–120 nm	Tetraspanins (such as TSPAN29 and TSPAN30), ESCRT components, PDCD6IP, TSG101, flotillin, MFGE8	mRNA, microRNA (miRNA) and other non-coding RNAs; cytoplasmic and membrane proteins including receptors and major histocompatibility complex (MHC) molecules
Microvesicles	Cell surface; outward budding of cell membrane	50–1,000 nm	Integrins, selectins, CD40 ligand	mRNA, miRNA, non-coding RNAs, cytoplasmic proteins and membrane proteins, including receptors
Apoptotic bodies	Cell surface; outward blebbing of apoptotic cell membrane	500–2,000 nm	Extensive amounts of phosphatidylserine	Nuclear fractions, cell organelles

ESCRT, endosomal sorting complex required for transport, MFGE8, milk fat globule-EGF factor 8 protein; PDCD6IP, programmed cell death 6 interacting protein (also known as ALIX); TSG101, tumour susceptibility gene 101 protein; TSPAN29, tetraspanin 29.

because of an as yet incomplete understanding of extracellular vesicle biogenesis, inconsistencies in extracellular vesicle purification protocols and a lack of thorough vesicle characterization. Here, we use the term 'extracellular vesicle' to refer to both of these vesicle types.

Biological roles of extracellular vesicles

Extracellular vesicles exert their effects on fundamental biological processes in a pleiotropic manner, directly activating cell surface receptors via protein and bioactive lipid ligands, merging their membrane contents into the recipient cell plasma membrane and delivering effectors including transcription factors, oncogenes, small and large non-coding regulatory RNAs (such as microRNAs (miRNAs)), mRNAs and infectious particles into recipient cells[1,4,5,33,41] (FIG. 1). In this way, extracellular vesicles participate in the maintenance of normal physiology — for example, stem cell maintenance[4], tissue repair[25], immune surveillance[21] and blood coagulation[26] (FIG. 2). Extracellular vesicles can thus be regarded as signalosomes: multifunctional signalling complexes for controlling fundamental cellular and biological functions.

For example, in the regulation of immune responses, depending on the status of particular immune cells, extracellular vesicles might trigger adaptive immune responses or suppress inflammation in a tolerogenic manner[42–50] (reviewed in REF. 23). Extracellular vesicles have been shown to confer immune suppression by several mechanisms: they can enhance the function of regulatory T cells[42], suppress natural killer (NK) and $CD8^+$ cell activity[43,44], and inhibit monocyte differentiation into DCs[45] as well as DC maturation[46]. By contrast, the effects of immune activation can be mediated by extracellular vesicle-promoted proliferation and survival of haematopoietic stem cells[47] and the activation of monocytes[48], B cells[49] and NK cells[50].

In the brain, in addition to classical synaptic neurotransmission, neurons communicate via the secretion of extracellular vesicles that can contribute to a range of neurobiological functions (including synaptic plasticity)[51] — for example, via the increased release of extracellular vesicles containing neurotransmitter receptors from cortical neurons following enhanced glutamatergic activity[52]. Extracellular vesicles have also been implicated in cell phenotype modulation — for example, in converting the haematopoietic stem cell phenotype into a liver cell phenotype[53] and in shifting the bone marrow cell transcriptome and proteome towards a lung phenotype *in vivo*[54–56]. Importantly, several reports have implicated extracellular vesicles in stem cell maintenance and plasticity, indicating that stem cell-derived extracellular vesicles have a pivotal role in tissue regeneration following injury[5,6,24].

Such wide-ranging cellular and biological functions indicate that extracellular vesicles, by virtue of their pleiotropic signalling effects, may have innate therapeutic potential — for example, in the fields of regenerative medicine and immunotherapy (FIG. 2) — as further discussed below.

Pathological roles of extracellular vesicles

Given their fundamental role in regulating biological processes, it is not surprising that in some contexts extracellular vesicles have an important role in disease pathogenesis. The best understood role of extracellular vesicles in disease is their role in tumour biology: numerous studies have implicated extracellular vesicles in driving the formation of a pre-metastatic tumour niche[27,57]. Extracellular vesicles are capable of stimulating tumour progression[5,27] via their ability to carry out the following processes: inducing proliferation in cells, thereby directly stimulating tumour growth[14,15]; stimulating angiogenesis[14–16]; promoting matrix remodelling via the secretion of matrix proteases[58]; inducing metastasis[57,58]; and promoting immune escape by modulating T cell activity[59–61]. Although the role of extracellular

vesicles in cancer has recently been comprehensively reviewed[5,27], key examples illustrating the role of extracellular vesicles in tumorigenesis are highlighted below.

Several years before extracellular vesicles were discovered to be involved in tumour spread, extracellular vesicles secreted by tumour cells were known to possess direct[62] or (activated platelet-mediated) indirect[26] procoagulant activity, linking cancer progression with extracellular vesicle-induced thrombosis[63]. A key study showing a direct link between extracellular vesicles and tumour invasion of healthy tissues was reported in 2008 by Al-Nedawi and colleagues[14]. This study elegantly demonstrated that the mRNA expression of an activated mutated epidermal growth factor receptor (EGFRvII) in glioma cells resulted in markedly enhanced vesiculation, which was detectable even in the blood of tumour-bearing mice, and intercellular transfer of this oncoprotein to adjacent tumour cells, leading to the production of angiogenic mediators such as vascular endothelial growth factor (VEGF)[14].

Similar results were reported by Breakefield *et al.*[15], showing that human primary glioblastoma cell-derived extracellular vesicles transfer not only EGFR but also various miRNAs that stimulate tumour growth and angiogenesis[15]. In a follow-up study by Al-Nedawi *et al.*[16], extracellular vesicles derived from tumour cells were shown to transfer activated EGFR to endothelial cells both *in vitro* and *in vivo*, subsequently inducing VEGF expression and leading to autocrine activation of VEGF receptor 2 to stimulate angiogenesis[16]. By inhibiting extracellular vesicle uptake through phosphatidylserine blockade, this study reported a marked reduction in tumour growth rate and microvascular density in mice with human carcinoma xenografts[16]. These results collectively suggest that extracellular vesicles can trigger tumour growth by stimulating the proliferation of cancer cells and by inducing angiogenesis in neighbouring endothelial cells, therefore interfering with this process can attenuate tumour progression.

The ability of extracellular vesicles to induce angiogenesis in endothelial cells has been confirmed in other studies; for example, extracellular vesicles from extracellular matrix metalloproteinase inducer (EMMPRIN; also known as Basigin, CD147 and TCSF)-positive ovarian cancer cells induce endothelial cell activation[64], and activated endothelial cells may communicate at a distance to propagate angiogenic signals

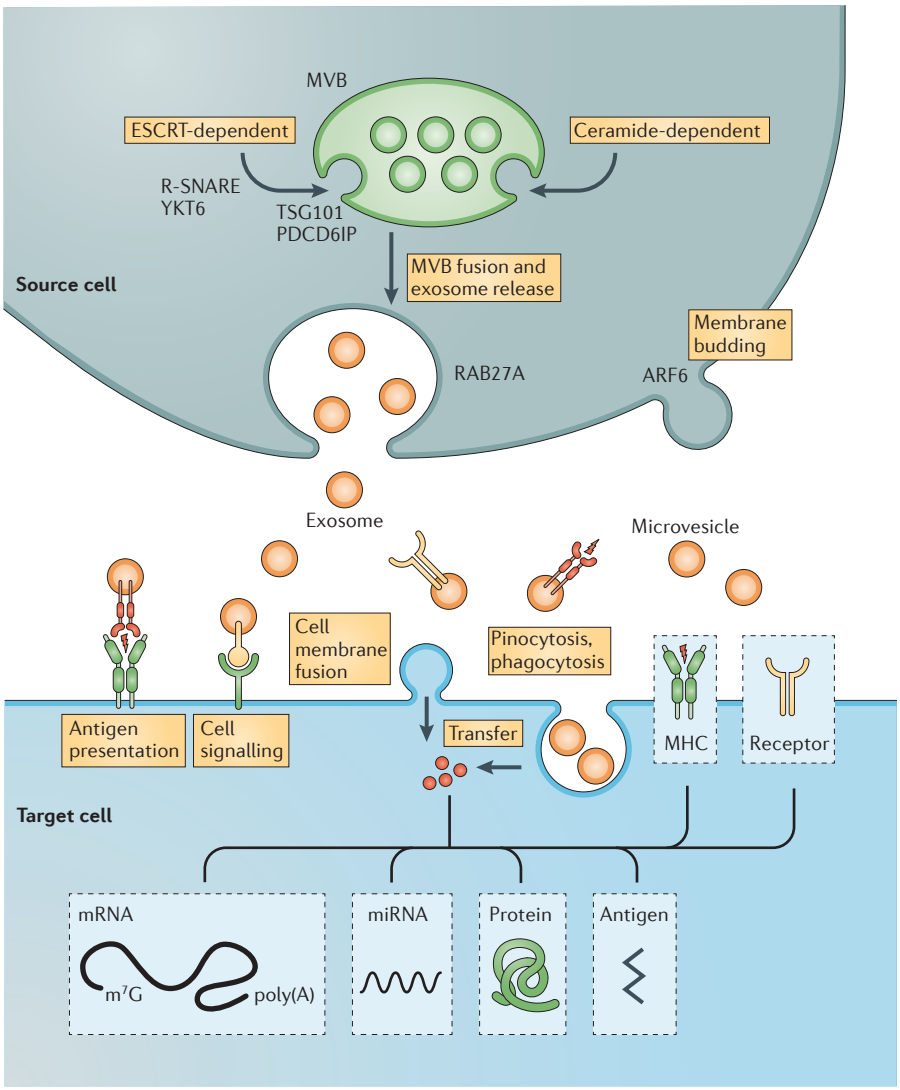


Figure 1 | **Biogenesis of extracellular vesicles and their interactions with recipient cells.** Exosomes are presumed to be a homogeneous population of vesicles of endocytic origin that are formed by the inward budding of the multivesicular body (MVB) membrane. Cargo sorting into exosomes involves the endosomal sorting complex required for transport (ESCRT) and other associated proteins such as programmed cell death 6 interacting protein (PDCD6IP; also known as ALIX) and tumour susceptibility gene 101 protein (TSG101)[38–40]. In addition to ESCRT, which recognizes ubiquitylated proteins, other ESCRT-independent mechanisms operate to generate exosomes of certain biochemical compositions (reviewed in REF. 22). For example, in some cells, exosome production requires the lipid ceramide and neutral sphingomyelinase — the enzyme that converts sphingomyelin to ceramide[70]. Exosomes are secreted following the fusion of MVBs with the cell membrane — a process that is, in some cells, dependent on small GTPases such as RAB27A, RAB11 and RAB31 (REFS 22,73). An alternative mechanism for the secretion of WNT-bound exosomes was recently shown to involve the SNARE (soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein (SNAP) receptor) protein YKT6 (REF. 117). Exosomes display the same membrane orientation as the cell of origin, similarly to microvesicles. Microvesicles, however, represent a relatively heterogeneous population of vesicles that are formed by the outward budding and fission of the cell membrane, which could be controlled by membrane lipid microdomains and regulatory proteins such as ADP-ribosylation factor 6 (ARF6)[118]. Extracellular vesicles can be regarded as signalosomes for several biological processes. They can be involved in antigen presentation and in the transfer of both major histocompatibility complex (MHC) molecules and antigens, thereby participating in immune regulation. Extracellular vesicles can directly activate cell surface receptors via protein and bioactive lipid ligands, transfer cell surface receptors or deliver effectors including transcription factors, oncogenes and infectious particles into recipient cells[5]. In addition, various RNA species including mRNAs and small regulatory RNAs (for example, microRNAs (miRNAs) and non-coding RNAs) are contained in extracellular vesicles and functionally delivered to recipient cells[1].

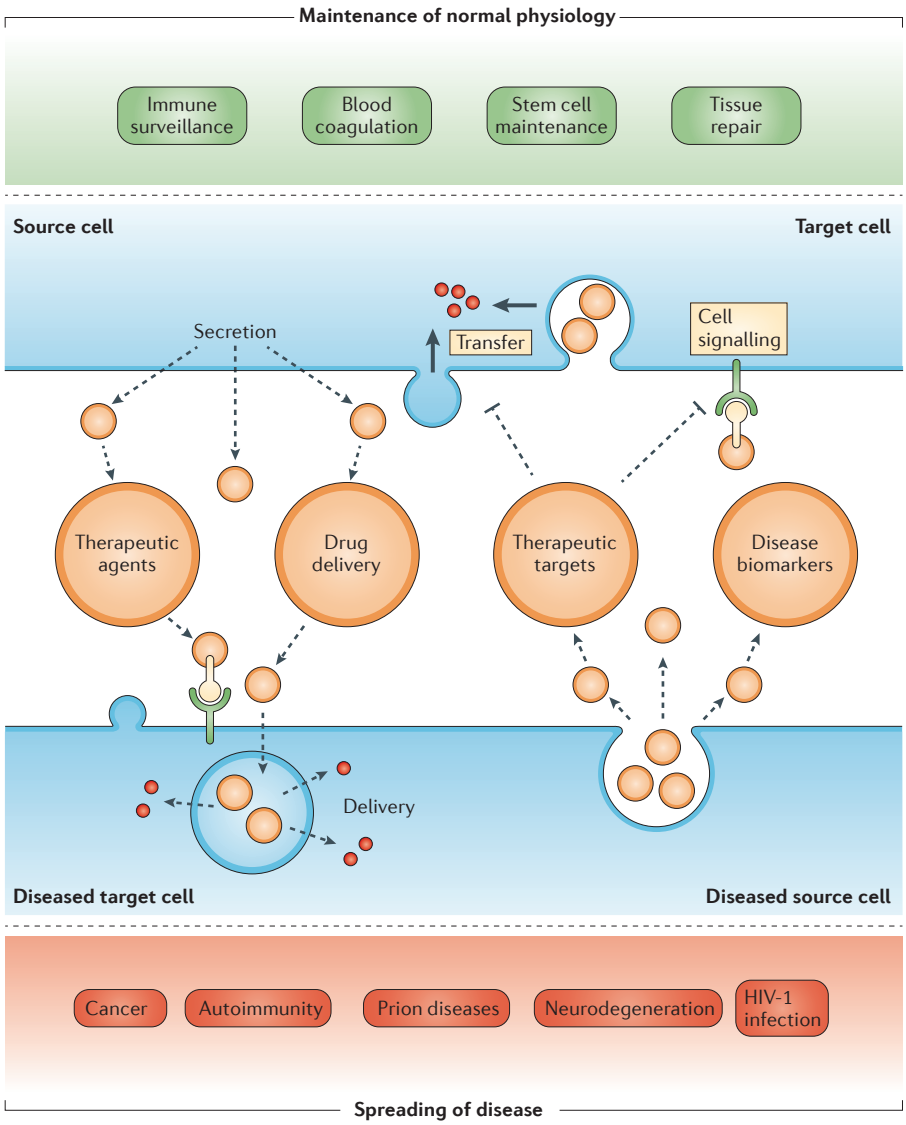


Figure 2 | **Roles of extracellular vesicles in normal physiology and disease pathogenesis.** Extracellular vesicles could be considered as signalosomes for several core biological processes. For example, extracellular vesicles may activate immune responses or suppress inflammation in a tolerogenic manner, thereby participating in immune surveillance. In blood circulation, extracellular vesicles participate in the coagulation cascade by providing a surface for the assembly of clotting factors. In the brain, neurons can communicate via the secretion of extracellular vesicles, which contribute to local and distal synaptic plasticity. Extracellular vesicles also take part in stem cell maintenance and plasticity, and they appear to have an essential role in the repair of injured tissue owing to their neoangiogenic, anti-apoptotic and cell proliferation-stimulating characteristics. These effects could be translated into therapies by using extracellular vesicles as therapeutic agents. Furthermore, the fact that extracellular vesicles are secreted by most cells, are rich in RNAs and are able to transfer their contents to recipient cells indicates that they would be highly suitable candidates for drug delivery, particularly therapeutic nucleic acid delivery. However, the same properties of extracellular vesicles that underline their important roles in the maintenance of normal physiology can lead to their involvement in pathological conditions. For example, extracellular vesicles can support tumour growth and tumour-related pathologies by inducing unwanted immune tolerance, spreading oncogenes (for example, MET), switching on angiogenic programmes and promoting metastases. In the case of the development of autoimmune disease, extracellular vesicles can induce immune responses toward self-antigens. Extracellular vesicle-mediated transfer of prion proteins and toxic protein aggregates can also modulate the progression of neurodegenerative diseases; furthermore, the transfer of extracellular vesicle-bound viral material has been implicated in HIV-1 infection. Owing to their involvement in disease progression, extracellular vesicles can be considered as targets for therapeutic intervention as well as useful disease biomarkers.

through the transfer of Delta-like 4 Notch ligand[65]. Further evidence supporting the involvement of tumour-secreted extracellular vesicles in the promotion of metastasis and tumour invasion includes the transfer of the EMMPRIN transmembrane glycoprotein, which stimulates matrix metalloproteinase (MMP) expression in fibroblasts and remodelling of the extracellular matrix[58]. Very recently, extracellular vesicles derived from melanoma cells were shown to educate bone marrow cells towards a pro-metastatic phenotype via the horizontal transfer of MET[57], further establishing their role in mediating communication between tumour cells and normal cells.

Interestingly, in addition to tumour-derived extracellular vesicles, tumour-associated macrophages can secrete extracellular vesicles enriched in certain miRNAs that promote the local invasion of breast cancer cells[66]. Some of the effects mediated by tumour-secreted extracellular vesicles are related to direct modulation of immune function. For example, extracellular vesicles can promote immune escape of tumours by inducing the expansion of regulatory T cells[59] and by shedding FAS ligand (FASL; also known as CD95L and TNFSF6), thereby inducing $CD8^+$ T cell apoptosis and increasing MMP9 expression in melanoma cells[60,61].

Collectively, these studies indicate that extracellular vesicles have an important and fundamental role in many steps leading to tumour progression. In addition, extracellular vesicles are implicated in tumour-associated pathologies such as thrombotic events[63]. Many cancer cells shed extracellular vesicles enriched in tissue factor[26] — the central component of the blood coagulation cascade. This, in combination with the fact that they expose phosphatidylserine, allows extracellular vesicles to provide a surface for the assembly of clotting components and thereby drive a prothrombotic process[5].

Beyond cancer, extracellular vesicles have been implicated in the spread of numerous pathogens, including: HIV-1, via the horizontal transfer of CC chemokine receptor 5 (CCR5), which is used for viral cell entry[28]; Epstein–Barr virus (EBV), via the transfer of viral miRNAs that repress the expression of EBV target genes in non-infected cells[67]; and prions, via the selective delivery of PrP with certain modifications and glycoforms into neuronal cells[28,31,67].

It is also likely that extracellular vesicles contribute to the local propagation of neurodegenerative disease. Neurons are known to communicate through the secretion of extracellular vesicles, which contribute to

local synaptic plasticity, but these extracellular vesicles also allow longer-range communication within the central nervous system and have an influence on static neuronal networks located at a distance[51]. This has been elegantly demonstrated in the context of Alzheimer's disease, in which amyloid-β peptides, the toxic protein species for this disease, have been shown to be released in association with exosomes, contributing to pathogenic amyloid-β deposition in other parts of the brain[29]. Similarly, α-synuclein protein has been detected within extracellular vesicles, which could provide a mechanism for the local propagation of Parkinson's disease from enteric neurons to the brainstem and higher cortical centres[30].

Inhibiting extracellular vesicles in disease

Given the growing evidence for extracellular vesicle-mediated disease pathogenesis, there are at least four strategies that could potentially be used to attenuate extracellular vesicle-driven disease that involve inhibiting various aspects of extracellular vesicle function. These include their biogenesis, release, cell uptake or the targeting of specific extracellular vesicle components that contribute to disease pathogenesis (FIG. 3a).

Inhibiting extracellular vesicle-mediated pathogenesis is of prototypical relevance in cancer, in which extracellular vesicles have been strongly implicated in many aspects of tumorigenesis and tumour-related pathology. It has been shown that the level of circulating extracellular vesicles increases more than twofold with cancer progression and correlates with survival in patients with melanoma[68]. Consequently, therapeutic interventions are being developed that are aimed at reducing the load of circulating extracellular vesicles or blocking crucial components of extracellular vesicles (FIG. 3a). However, caution is warranted at this stage given that most *in vitro* and *in vivo* studies to date investigating the pathological role of extracellular vesicles have been carried out using high concentrations of extracellular vesicles, sometimes exceeding the numbers normally found in circulation[69]. Potential therapeutic interventions (FIG. 3a) are described below.

Inhibiting extracellular vesicle formation. Various cellular components are known to be crucial for the formation of extracellular vesicles but specific inhibition strategies are in early stages of development and are largely untested in disease models. However, inhibition of ceramide formation (which is important in endosomal sorting and exosome biogenesis)[70] using small-molecule inhibitors of neutral sphingomyelinase or via treatment with the blood-pressure-lowering drug amiloride (which generally attenuates endocytic vesicle recycling) can reduce extracellular vesicle yields[70,71]. The latter strategy (the use of amiloride) has proven to be efficacious *in vivo* in reducing mouse and human tumour cell growth by blocking the secretion of tumour-derived extracellular vesicles harbouring membrane-associated heat shock protein 72 (HSP72), which otherwise mediates immunosuppressive effects on myeloid-derived suppressor cells[71].

Importantly, a recent study elegantly demonstrated the importance of syndecan proteoglycans and their cytoplasmic adaptor syntenin, which directly interacts with the exosomal protein programmed cell death 6 interacting protein (PDCD6IP; also known as ALIX), in regulating exosome formation[39]. Directly interfering with this interaction — either by RNA interference (RNAi) or using small-molecule inhibitors — could thus attenuate exosome release. An alternative but as yet untested approach could be to sterically block specific tetraspanins (such as tetraspanin 30 (TSPAN30; also known as CD63)), which are important for extracellular vesicle formation and involved in tumorigenesis[72].

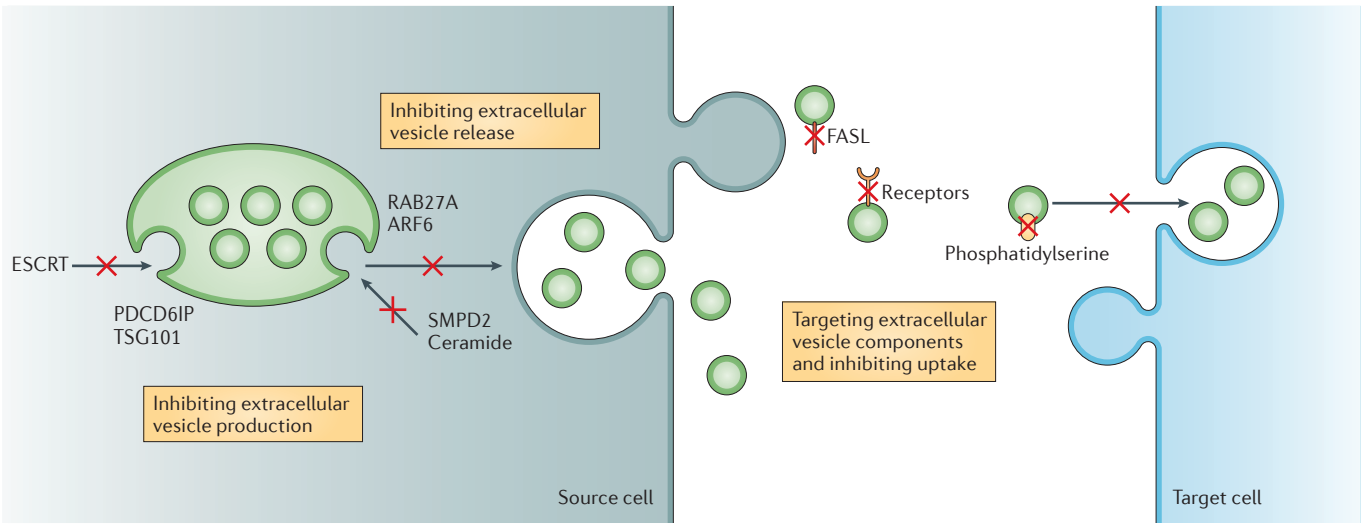
Inhibiting extracellular vesicle release. Many proteins have been implicated in the secretion of extracellular vesicles, but the precise mechanism of regulated extracellular vesicle release remains elusive and is likely to vary among different cells. However, in some tumour cells, exosome release depends on the small GTPase RAB27A[73], and this was demonstrated to be a plausible therapeutic target (using RNAi) for reducing tumour exosome-mediated signalling to suppress neutrophils that support tumour growth[74]. This approach reduced the growth rate of primary metastatic carcinoma and reduced metastasis to the lungs in mice[74]. These findings were recently corroborated in an independent study in a mouse model of melanoma *in vivo*[57], in which inhibition of RAB27A led to a reduction in tumour growth and metastasis principally by preventing the reprogramming of bone marrow progenitor cells towards a pro-metastatic and pro-vasculogenic phenotype. Other GTPases such as RAB11 and RAB35 might serve as alternative targets for inhibiting the release of exosomes by impairing the docking and/or fusion of multivesicular bodies with the plasma membrane[75,76].

Inhibiting extracellular vesicle uptake. Several uptake mechanisms have been proposed for extracellular vesicles, but there is a lack of detailed knowledge regarding the key steps in extracellular vesicle trafficking and target definition. Nevertheless, the uptake of extracellular vesicles released from tumour cells can be attenuated by blocking surface phosphatidylserine — which is important for cell adhesion — using diannexin[16,77]. Indeed, this strategy was shown to reduce the growth rate of human glioma xenografts *in vivo* in mice. However, although feasible, the widespread applicability of this strategy could be hampered by its limited specificity in relation to other physiological functions of phosphatidylserine: for example, in triggering the clearance of apoptotic cells. In the non-tumour context, dissemination of HIV-1 to T cells could be attenuated by targeting intercellular adhesion molecule 1 (ICAM1), which is displayed on extracellular vesicle-encapsulated viruses, thus preventing binding to a specific integrin: β2 integrin (also known as LFA1) (reviewed in REF. 17). As aforementioned, another proposed mechanism of HIV-1 dissemination to non-haematopoietic cells is by the horizontal transfer of chemokine receptors via extracellular vesicles, which makes these vesicles attractive targets for intervention as well[28].

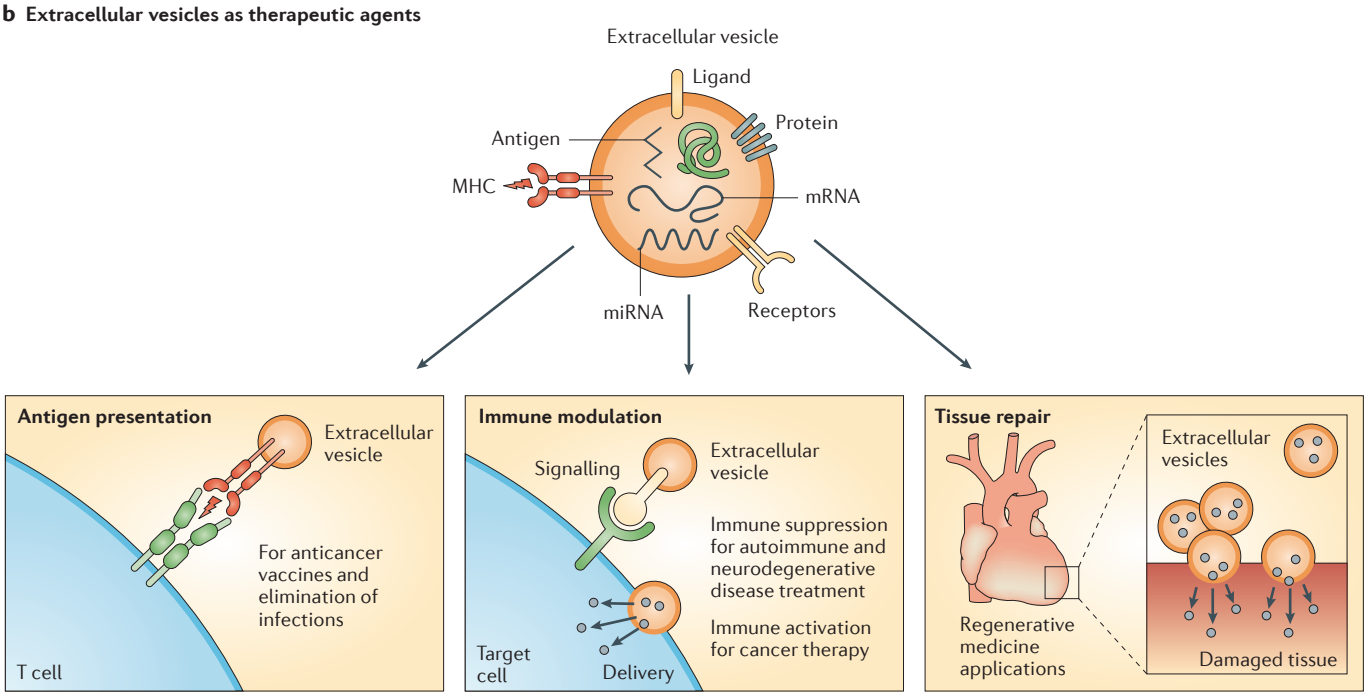
Blocking specific extracellular vesicle components. Blocking specific signalling components of extracellular vesicles could prove to be therapeutically relevant. For example, it has been shown that FASL-specific monoclonal antibodies targeting FASL1 displayed on extracellular vesicles reduce tumour growth in a melanoma model[61]. However, this strategy may lack specificity and negatively affect global immune function. Similarly, the targeting of MET by RNAi to prevent its active incorporation into extracellular vesicles could prove to be useful in reducing metastasis in late-stage melanoma[57], provided that sufficiently effective small interfering RNA (siRNA) delivery to tumour cells can be achieved *in vivo*.

The various approaches highlighted above are attractive and likely to prove tractable for the development of small-molecule therapeutics. However, it is important to emphasize that interfering with aspects of extracellular vesicle biogenesis could result in undesirable off-target effects not only because extracellular vesicles are important for the regulation of normal biological processes but also because many of the proteins that are implicated in extracellular vesicle biogenesis are important in several

a Extracellular vesicles as therapeutic targets



b Extracellular vesicles as therapeutic agents



c Extracellular vesicles for drug delivery

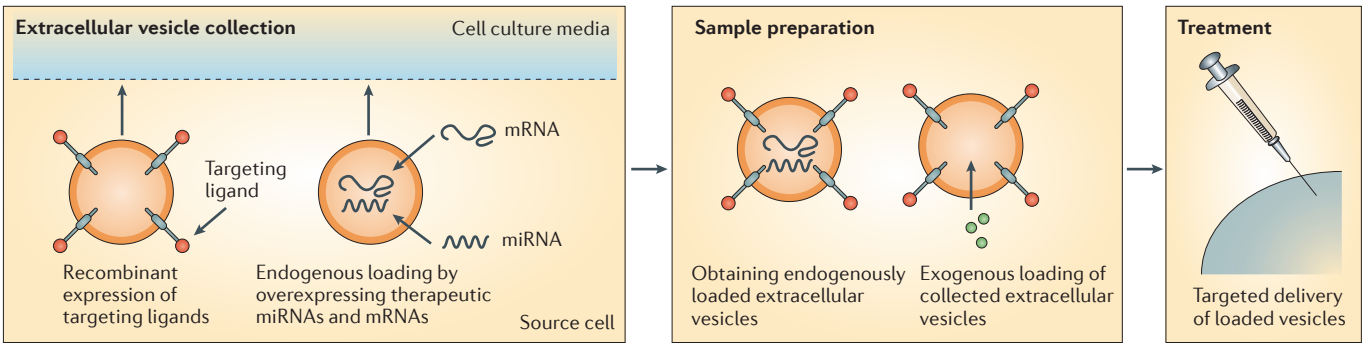


Figure 3 | **Therapeutic targeting and exploitation of extracellular vesicles. a** | The fact that extracellular vesicles are involved in many pathological conditions indicates that disease progression could be alleviated by specifically inhibiting the production or release of extracellular vesicles, or by targeting extracellular vesicle components and inhibiting their uptake. Extracellular vesicle production can be lowered by blocking ceramide formation in cells, by treating producer cells with amiloride, by interfering with syntenin–syndecan interactions or by blocking tetraspanins. Some studies indicate that the release of extracellular vesicles could be reduced by blocking certain RAB GTPases or ADP-ribosylation factor 6 (ARF6). Alternatively, it could be advantageous to block certain extracellular vesicle-bound receptors or lipids that mediate either extracellular vesicle uptake or signalling through cell surface receptors. Some of these strategies have been tested, providing proof-of-concept evidence for their potential feasibility. Notably, some of the presented examples may not be specific towards extracellular vesicle-mediated effects but nevertheless indicate the principal feasibility of these types of strategies. **b** | As extracellular vesicles participate in the modulation of normal physiological processes, they could be used as therapeutic agents in regenerative medicine and tissue repair as well as in the modulation of immune responses in a desired direction either by antigen-specific or -unspecific events. Tissue repair can depend on the exosomal transfer of growth factors, soluble proteins, bioactive lipids and genetic material, such as microRNA (miRNA), mRNA and non-coding RNA[24,83,86,87], as shown in models of myocardial infarction and ischaemia–reperfusion injury, acute kidney injury studies, during neoangiogenesis of pancreatic islet cells and in skeletal muscle repair. The immunomodulatory effects of extracellular vesicles can include antigen transfer and presentation: for example, as used in anticancer vaccines and in the elimination of infections by priming specific $CD8^+$ T cells. Overall immune activation properties can be important in cancer therapy and other immunotherapy applications, as extracellular vesicles may promote the release of pro-inflammatory cytokines from target macrophages. Furthermore, extracellular vesicles can enhance natural killer (NK) cell activity and overall T cell survival. The effects of extracellular vesicles on the immune system can also be suppressive, depending on the vesicle type. For example, extracellular vesicles can inhibit overall T cell proliferation, NK cell activity and dendritic cell (DC) differentiation. Also, exosomal interleukin-10 (IL-10) and FAS ligand (FASL) signalling can induce T cell apoptosis and favour the growth of regulatory T cells and myeloid suppressor cells. This can be important in the context of autoimmune and neurodegenerative diseases as well as systemic inflammation. **c** | As extracellular vesicles naturally contain mRNA and regulatory RNA, they can be utilized for delivering oligonucleotide drugs of choice. Extracellular vesicles can be engineered to have certain tissue- or cell-type-specific targeting ligands present on their surface by expressing plasmid fusion constructs comprising targeting ligands fused to extracellular vesicle transmembrane proteins, such as lysosomal-associated membrane protein 2 (LAMP2)[35] in cells. Drug loading can be carried out either endogenously or exogenously. Endogenous loading is achieved by strongly overexpressing miRNA, short hairpin RNA or mRNA in source cells, resulting in extracellular vesicles that are already loaded with the drug upon their collection. Exogenous loading allows the collection of (drug-free) extracellular vesicles before their loading with desired cargo molecules either by simple co-incubation with suitable cargo molecules such as curcumin[36] or with the help of certain manipulations, such as electroporation[34,35]. The collection and purification of extracellular vesicles can be carried out by different methods, including differential ultracentrifugation, ultrafiltration, sucrose gradient centrifugation, immunoprecipitation, high-performance liquid chromatography, and so on[91,119,120]. Extracellular vesicles, loaded by any of these strategies, can then be tested in cell culture or *in vivo*. ESCRT, endosomal sorting complex required for transport; MHC, major histocompatibility complex; PDCD6IP, programmed cell death 6 interacting protein (also known as ALIX); SMPD2, sphingomyelin phosphodiesterase 2 (also known as neutral sphingomyelinase 2); TSG101, tumour susceptibility gene 101 protein.

other core cellular processes. Moreover, such approaches are likely to require a drug delivery system that is capable of targeting specific extracellular vesicle-producing cell populations.

Therapeutic extracellular vesicles

By virtue of their bioactive cargoes, extracellular vesicles have innate therapeutic potential, in particular for driving tissue regeneration (FIG. 3b). This is because extracellular vesicles that are derived predominantly from stem cells have the ability to induce angiogenic programmes in quiescent endothelial cells[78], suppress apoptosis and stimulate cell proliferation[25,79], deliver immunomodulatory signals[21] as well as recruit and/or reprogramme cells that are required for tissue regeneration[4]. By contrast, extracellular vesicles that are derived from specific differentiated cell types (for example, from immunomodulatory immune cells) could be exploited to induce or inhibit specific immune responses.

In regenerative medicine, the application of multipotent stem cells typically isolated from the bone marrow or peripheral blood — such as MSCs and haematopoietic stem cells — is being investigated. The therapeutic potential of these cells, particularly MSCs, is reflected in the growing number of ongoing clinical trials for various diseases, including cardiovascular disease, graft versus host disease and Crohn's disease, as well as in acute kidney injury (see the ClinicalTrials.gov website).

The success of MSC-based therapy was established based on the premise that transplanted cells home in to and engraft within injured tissues and subsequently differentiate to replace injured cells. However, following myocardial infarction, most transplanted MSCs accumulate in tissues other than the heart, and transdifferentiation into cardiomyocytes is a relatively slow and inefficient process, whereas the observed restoration of heart function is more rapid[6,80]. These findings led to the hypothesis that soluble factors released by MSCs are responsible for the beneficial outcomes: the so-called paracrine effect. This hypothesis was confirmed in the mid-2000s when it was shown that conditioned media from hypoxic MSCs could limit infarct size and improve heart function[81,82]. Similar results have been obtained in both pigs[7] and mice[8] in models of myocardial ischaemia–reperfusion injury as well as in a model of kidney injury[83].

Subsequent work has affirmed that the paracrine effect is mediated by secreted extracellular vesicles, particularly exosomes[84,85], not only in models of myocardial infarction but also in several other models of injury such as kidney injury and skeletal muscle repair. Moreover, the paracrine effect depends on the transfer — via extracellular vesicles — of not only growth factors but also other proteins, bioactive lipids and genetic material including mRNA, miRNA and other non-coding RNA[24,83,86,87]. Furthermore, extracellular vesicles derived from other cell sources, in particular from endothelial progenitor cells, have been demonstrated to protect the kidney from ischaemia–reperfusion injury and to enhance the neoangiogenesis of human pancreatic islet cells[88,89].

This paracrine hypothesis also suggests that certain safety concerns related to stem cell transplantation, such as aberrant stem cell differentiation, cardiac arrhythmia or vascular occlusion, could be readily overcome using stem cell-derived extracellular vesicles in the absence of the stem cells of origin. Advances have already been made towards the translation of MSC-derived extracellular vesicle regenerative therapy by establishing immortalized MSCs and large-scale production protocols[87,90]. However, further progress will require detailed

optimization of cell types for the production, culture, purification and characterization of extracellular vesicles, as well as longer-term safety studies before clinical translation[91]. The essential feature that makes MSCs and their extracellular vesicles attractive for therapy is their availability from patients' bone marrow and their ability to suppress inflammation, which is pivotal for subsequent tissue regeneration and for allowing them to be exploited effectively in an allogeneic setting.

The innate ability of extracellular vesicles to modulate immune responses can also be exploited for immunotherapy, particularly for cancer vaccination but also in the treatment of infectious diseases via the priming of specific $CD8^+$ T cell subsets. In 1996, Raposo *et al.*[21] showed that both human and murine B lymphocytes secrete exosomes that carry major histocompatibility complex (MHC) class II molecules in a peptide-bound conformation; furthermore, they showed that these extracellular vesicles could induce MHC class II restricted T cell responses[21]. Concomitantly, mouse DCs were demonstrated to secrete exosomes expressing both class I and class II MHC molecules, which — when pulsed with tumour-related peptides — were found to suppress tumour growth in mice in a T cell-dependent manner[32]. Since these two pioneering reports, there has been a rapid increase in the number of studies investigating the role of extracellular vesicles in immune system modulation (such as antigen presentation, antigen transfer, antigen nonspecific inhibition and the promotion of immune responses).

The immune properties of extracellular vesicles have been reviewed recently by Thery and colleagues[17]; here, we highlight important examples (FIG. 3b). Extracellular vesicles can participate in the activation of the immune system and augmentation of antigen presentation[17] by inducing the release of pro-inflammatory cytokines from macrophages[92], increasing the release of tumour necrosis factor from macrophages[93], enhancing NK cell activity[94] and protecting T cells from activation-induced cell death[95]. Conversely, depending on the source of the extracellular vesicles, their immunosuppressive properties can be exploited — for example, in the treatment of autoimmune diseases[17]. Such immunosuppressive effects can emanate from the ability of certain extracellular vesicles to induce FASL-mediated T cell apoptosis[96], inhibit T cell proliferation[42], reduce NK cell activity or DC differentiation[45], favour the expansion of T_{Reg} cell subsets[97] and myeloid suppressor cells[45] and/or inhibit inflammation via exosomal IL-10 delivery[96].

> "Targeting extracellular vesicles to inhibit their effect in disease, exploiting their innate therapeutic potential or using them for drug delivery are all emerging important strategies for therapy."

Extracellular vesicles for drug delivery

Potentially the most intriguing feature of extracellular vesicles is their ability to transmit various RNA species among cells. Early studies from the Ratajczak group[4,41] identified mRNAs in extracellular vesicles derived both from embryonic stem cells and cancer cells, and showed that these could be transferred to haematopoietic progenitor cells and monocytes, respectively, to induce phenotypic changes[4,41]. Subsequently, extracellular vesicles derived from endothelial progenitor cells were shown to activate angiogenic pathways through the horizontal transfer of mRNA associated with the phosphoinositide 3-kinase (PI3K)–AKT signalling pathway[78]. This was followed up by two very convincing studies showing that extracellular vesicles not only harbour mRNAs but could also be delivered to recipient cells and translated into protein.In the first such study, human mast cell-derived exosomes delivered mRNAs that were expressed in mouse mast cells[33], and in the second study, glioma cells were shown to secrete microvesicles bearing a reporter mRNA that was subsequently expressed in recipient cells[15]. Importantly, in both studies, high amounts of smaller RNA species — particularly miRNAs — were detected inside extracellular vesicles. These results have been corroborated in several subsequent reports identifying miRNAs in extracellular vesicles derived from MSCs[98], tumour cells[99], T cells[100], DCs[101] and EBV-infected B cells[67].

Importantly, it has been elegantly demonstrated that extracellular vesicles contain several proteins and ribonucleoproteins that are involved in RNA transport and RNA processing, including the double-stranded RNA-binding protein Staufen homolog 1 (STAU1), STAU2, Argonaute 2 (AGO2) and its interacting partner trinucleotide repeat-containing gene 6A protein (TNRC6A; also known as GW182), suggesting that RNA compartmentalization is dynamically regulated in extracellular vesicles[98,102,103]. The fact that extracellular vesicles are secreted by most cells, are rich in RNAs and are able to transfer their RNA content to recipient cells indicates that they may represent highly suitable candidates for drug delivery, particularly therapeutic nucleic acid delivery. Such a strategy is envisioned in FIG. 3c.

Non-viral delivery vectors have been developed to improve the bioavailability of the nucleic acid-based drugs that are used to interfere with gene expression in different disease contexts. However, such vectors typically have the inherent risks of inducing immune activation owing to their foreign nature and systemic toxicity; furthermore, to date, these vectors have only proven to be efficacious for liver- or tumour-targeting strategies (reviewed in REF. 104). By contrast, extracellular vesicle-mediated delivery offers several advantages: these vesicles are biocompatible, immunologically inert if they are derived from appropriate cells (for example, MSCs or immature DCs), can be patient-derived if required and have an innate ability to cross major biological barriers including the blood–brain barrier (BBB)[35].

The first report on extracellular vesicle-mediated transfer of exogenous nucleic acids was published in 2010, when it was shown that THP-1 monocytes transfected with a miR-150 mimic secreted extracellular vesicles enriched in miR-150, which was functionally delivered to recipient cells[103]. Similarly, Akao *et al.*[105] demonstrated that when injected intravenously into nude mice, THP-1 cells that had been transfected *ex vivo* with a miR-143 mimic secreted miR-143-containing extracellular vesicles. Other studies have confirmed such modes of nucleic acid transfer. For example, hepatic cells or MSCs transduced with shRNA-expressing plasmids were shown to promote shRNA-mediated RNAi responses when co-cultured with recipient cells[106,107].

The Wood laboratory recently harnessed the RNA-transporting capacity of exosomes, providing the first evidence for the delivery of exogenous siRNA using such vesicles[35]. Given the lack of delivery vectors that can effectively traverse the BBB, the study set out to target exosomes to the brain. Immature DCs were transfected with a plasmid expressing an exosomal protein (lysosomal-associated membrane protein 2 (LAMP2; also known as CD107b)) fused with a brain-specific peptide (rabies virus glycoprotein (RVG)-derived peptide)[108] displayed on the exosome surface. Targeted exosomes were subsequently loaded with siRNA by electroporation and systemically delivered to mice. Strong RNAi responses were observed throughout the brain with, surprisingly, only

minor RNAi effects detected in the liver and spleen. Importantly, the treatment displayed minimal toxicity and immune stimulation, even following repeated administration. A subsequent study using siRNA-loaded exosomes derived from human plasma confirmed the potential of exosomes for siRNA delivery. Non-targeted exosomes efficiently promoted siRNA-mediated RNAi responses in both monocytes and lymphocytes[34], the latter being very difficult to transfect by conventional means.

More recently, the Breakefield laboratory reported two different strategies to exploit extracellular vesicles for gene therapy. In the first study, adeno-associated viruses (AAVs) encapsulated in extracellular vesicles displaying viral capsid proteins, aptly named vexosomes, were shown to be substantially more efficient than free AAVs for the delivery of genetic cargo into recipient cells. Gene delivery was dependent on the vexosomal AAV transgene and not extracellular vesicle-bound mRNA or protein transfer, as the elimination of viral capsid proteins from extracellular vesicles eradicated delivery of the cargo[109]. These authors have also identified a potential 'zipcode' sequence in the 3′ untranslated region of mRNAs that can lead to their enrichment in extracellular vesicles[110]. In another study, extracellular vesicles harbouring suicide gene mRNA and protein were derived from pre-transfected parental cells. These extracellular vesicles were subsequently used to treat Schwannoma tumours in an orthotopic mouse model, and resulted in reduced tumour growth[111]. Targeting exosomes more specifically to tumours could further enhance the anticancer properties associated with extracellular vesicles, as demonstrated in a study in which miRNA-loaded extracellular vesicles homed in to EGFR-expressing tumour cells via a peptide-targeting ligand[112].

Although they are at an early stage, these studies collectively emphasize the potential of exploiting extracellular vesicles for the therapeutic delivery of nucleic acids. The fundamental property that makes extracellular vesicles ideal drug delivery vehicles is their inherent ability to cross biological barriers, even the BBB. This BBB-penetrating capacity, which was initially reported in the Alvarez-Erviti[35] study, was corroborated in a subsequent study in which naked extracellular vesicles loaded with small-molecule drugs were shown to cross the BBB following intranasal delivery and were also shown to promote drug-mediated biological responses in mice[36]. This study highlights the wider potential of extracellular vesicles, beyond nucleic acid delivery, for the transfer of various other therapeutic cargoes. Before the full potential of extracellular vesicles for macromolecular drug delivery can be realized, a greater range of potential drug cargoes must be explored and drug-loading procedures optimized and standardized. Evaluating the different cell types from which extracellular vesicles can be derived for drug delivery, as well as optimizing tissue-targeting and barrier-crossing motifs, other than the RVG peptide used in the Alvarez-Erviti study[35], should further enhance the potency of delivery to desired tissues (FIG. 3c).

Translation into clinical use

Unarguably, extracellular vesicles have emerged as biological agents that are central to intercellular communication and have therapeutic potential. However, despite intense investigations during the past decade aimed at elucidating extracellular vesicle biology, many properties and mechanisms currently remain elusive. In fact, some reports describe contradictory results, even with extracellular vesicles derived from the same cell types. For example, MSC-derived exosomes have been shown to both inhibit and promote tumour growth[113,114]. Such discrepancies are probably a consequence of differences in cell culture conditions before extracellular vesicles are harvested, differences in the purification protocols used or due to a lack of robust extracellular vesicle characterization (FIG. 3c). It is clear that ambient cell culture conditions influence the content of extracellular vesicles[115]. For example, in the context of tissue regeneration, it appears to be crucial that parental cells are grown under hypoxic conditions in order to maximize the immunomodulatory and regenerative properties of derived extracellular vesicles[82]. Inducing cell stress is also known to substantially increase the production of extracellular vesicles[116].

Furthermore, to delineate the biological roles and therapeutic potential of extracellular vesicles, standardized protocols for their purification are urgently needed. Differential ultracentrifugation has been the gold standard for exosome purification; however, there are concerns relating to the relatively low yield and activity of vesicles[6]. Instead, filtration-based methods followed by liquid chromatography-based separation are emerging as viable alternatives that allow for the large-scale production of intact extracellular vesicles, and it could be necessary to develop further alternative procedures. Such developments are timely, as preclinical and clinical trials will require large-scale, cost-effective and standardized protocols for the production of extracellular vesicles.

Finally, more rigorous characterization of extracellular vesicles using a combination of methods — including sucrose gradient separation, electron microscopy and full RNA, lipid and protein profiling — will be required to fully explore the biology of extracellular vesicles and to assess potential biohazards. The latter will include, for example, mitigating the potential risks of therapeutic exosome transfer with respect to the cell type of origin, undesirable genetic and/or protein component transfer, deleterious immune system activation and animal virus transmission before clinical application.

We also envision that combinatorial extracellular vesicle-based therapeutics will be developed in the future. For example, MSC-derived extracellular vesicles, or extracellular vesicles derived from immature DCs pulsed with IL-10 — which are both inherently immunosuppressive — could be loaded with anti-inflammatory molecules, thereby combining their innate immunomodulatory properties with the ability to deliver a synergistic drug. Such extracellular vesicles could be evaluated for the treatment of acute or chronic inflammatory conditions such as graft versus host disease or Crohn's disease. Similarly, DC-derived extracellular vesicles presenting tumour antigens to T cells could be used in conjunction with tumour-targeted extracellular vesicles loaded with RNAi effectors to target components of exosome biogenesis, such as RAB27A. This approach encompasses all three therapeutic strategies relating to extracellular vesicles: innate potential as a cancer vaccine; drug delivery via siRNA; and targeting of extracellular vesicle-mediated pathogenesis (by RAB27A knockdown).

Conclusion

Extracellular vesicles are important conveyers of information between cells, through the transmission of various proteins, bioactive lipids and genetic information to alter the phenotype and function of recipient cells. Thus, extracellular vesicles have now been implicated in numerous biological and pathological processes. Targeting extracellular vesicles directly to inhibit their deleterious effects in mediating disease or exploiting their inherent potential to stimulate regenerative responses or to deliver nucleic acids and other drug cargoes across major biological barriers are emerging as important novel therapeutic strategies.

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Competing interests statement

The authors declare competing financial interests: see Web version for details.

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