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Challenges and directions in studying cell–cell communication by extracellular vesicles

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Abstract
Extracellular vesicles (EVs) are increasingly recognized as important mediators of intercellular communication. They have been shown to have important roles in numerous physiological and pathological processes, and show considerable promise as novel biomarkers of disease, therapeutic agents, and drug delivery vehicles. Intriguingly, however, understanding of the cellular and molecular mechanisms that govern the many observed functions of EVs remains far from comprehensive, at least partly due to technical challenges in working with these small messengers. Here, we highlight areas of consensus, as well as contentious issues, in our understanding of the intra- and intercellular journey of EVs: from biogenesis, release and dynamics in the extracellular space, to interaction with, and uptake by, recipient cells. We define knowledge gaps, identify key questions and challenges, and make recommendations on how to address these.

[H1] Introduction

Extracellular vesicles (EVs) are membrane-bounded particles released by cells into the extracellular space. Although evidence for their existence has been documented over 80 years, only in the last few decades have the pathways of their generation, functions and potential applications started to emerge. The number of EV-related publications has increased exponentially in recent years, and our understanding of EV biology has increased immeasurably. They are now known to bear many biological functions and are implicated in several pathologies. EVs also have tremendous potential as biomarkers, as therapeutic agents and as vehicles for therapeutic molecules. There is now broad consensus that EVs

are part of the intercellular signalling network that takes place in a multicellular organism[1]. However, although there are many areas of consensus regarding mechanisms of cell–cell communication via EVs, as with any rapidly growing field, there remain several challenges, and areas of disagreement.

The purpose of this article is to outline aspects of EV biology that are widely agreed on, highlight areas that are the most contentious, and propose areas that are in particular need for further research. Our intention is not to provide a comprehensive evaluation of the literature, and the references used throughout are only meant to serve as examples. To provide a framework for this endeavour we will describe the 'journey' of EVs, from biogenesis and release from a donor cell to their uptake and usage/function in a recipient cell. The biogenesis of EVs is relatively well described, though much detail remains to be elucidated, particularly how the different mechanisms of EV production are utilized to generate a heterogeneous array of vesicles[2-4]. The means of EV uptake are also relatively well explored, with a number of different pathways implicated in the bulk internalisation of vesicles[2,5]. The biggest unknowns, at this time, are the details of how the EVs are able to traverse the physical gap between donor and recipient cells, and how, once internalised, the cargoes of the EVs are delivered in such a manner that they can be functionally utilised. Moreover, it needs to be highlighted that horizontal transfer of biological cargo should not always be seen as the main function of EV secretion, and other EV functions (including signalling at the recipient cell surface, trophic support, clearance of obsolete cellular material and modulation of interstitial fluid or extracellular matrix) should be considered, but these remain relatively understudied. These gaps in our knowledge are largely due to the many challenges associated with working on EVs. The field faces an insufficiency in tools and models to study these messengers, owing to their often nanoscopic size, the poorly understood heterogeneity of EV populations, and low molecular cargo copy number per vesicle all leading to considerable challenges in their isolation and analyses. As with other emerging fields we also face a healthy debate on the interpretation, variability and reporting of data, and disagreements even on the nomenclature and definitions used to describe EVs. Herein we propose he paths the field should take to reach a more complete picture of the EV journey, hoping to eventually galvanise our collective efforts to achieve this goal.

Commented [PS1]: Au: What do you mean by copy number? Are you referring to cargo here?

Commented [MOU2]: Yes we do. maybe another way to say it ?

[H1] Extracellular vesicle biogenesis

EVs comprise a heterogeneous population of membrane vesicles that are generated via diverse mechanisms (Fig. 1; Table 1). The two main EV subpopulations include ectosomes and exosomes. The former comprises diverse types of EVs such as **oncosomes** **[G]** and microvesicles that are generated at the plasma membrane from its outward budding. Exosomes are produced inside the cell, within the endocytic pathway, by inward budding of the endosomal membrane, which results in the formation of very small vesicular structures contained within the endosome lumen. These intraluminal vesicles (ILVs) can represent pre-secreted exosomes that may be expelled into the pericellular space upon fusion of these so-called multivesicular endosomes (MVEs; also known as multivesicular bodies (MVBs)) with the plasma membrane, although not all ILVs may have this fate (reviewed in [3]). Understanding how different EV subtypes are generated has been a long-standing goal as it is important to discriminate them, to define their respective physiological relevance, to modulate their production in pathophysiological conditions and also to manipulate them as therapeutics. Below we highlight key areas associated with EV biogenesis that require further investigation to better understand the process of EV release (see also Box 1).

Commented [MOU3]: "a majority of ILVs may not have this fate" may better reflect the reality

[H2] Discrimination of extracellular vesicle subtypes
The site of biogenesis of EVs, i.e. plasma membrane or MVEs, is a first basis for the establishment of a nomenclature that will distinguish ectosomes from exosomes. Present

isolation strategies for EVs allow classification according to size, density or surface antigen, but do not discriminate EVs based on their site of biogenesis and thus preclude their identification as exosomes or ectosomes as these populations show overlap in terms of composition and size[6]. As a result, it is common for researchers to rely on the general and all-inclusive term of 'extracellular vesicles' to avoid misinterpretation, or incorrect definitions, at the risk of this term at times being insufficiently explicit and too vague. Only further research will teach us whether the classification of EVs according to their size and density is relevant and sufficient in order to understand their other properties and functions, or whether we need to distinguish EVs also according to their subcellular origin. The field is still awaiting optimal methods for isolation, separation and characterization of the different subpopulations of EVs that would facilitate establishment of a more accurate and specific nomenclature.

Commented [MOU4]: "respective" instead of "other"

[H2] Diversity of biogenesis machineries, EV subtypes and their cargoes

The mechanisms involved in the biogenesis and release of these EV subpopulations are relatively well understood (Fig. 1a) as compared to other aspects of the EV journey, certainly because the field has benefited from existing knowledge on mechanisms of intracellular membrane and protein trafficking that are related to the biogenesis of EVs[3,7]. Nevertheless, multiple facets of these processes remain unresolved.

The biogenesis of EVs generally relies on basic steps that are shared by various intracellular trafficking processes elsewhere in the cell (for example, during endocytosis at the plasma membrane): generation of membrane microdomain enriched in specific cargoes (e.g. proteins, lipids) and then budding and fission of the microdomain to generate a vesicle. Various machineries have been described to regulate each of these steps, including the **ESCRT machinery** **[G]** [8], the **syntenin-Alix pathway** **[G]** [9], **tetraspanins** **[G]** [10], the cytoskeleton[11], lipids[12,13], and **arrestin domain-containing protein 1** **[G]** (ARRDC1)[14]. These sorting machineries enrich specific sets of cargoes into EVs and their depletion can abrogate the generation of a defined EV subpopulation. Membrane cargoes, through their expression *per se* or by post-translational modification (e.g. ubiquitylation, cleavage, palmitoylation) recruit distinct sorting machineries at the plasma membrane and in endosomes and the overexpression of specific cargoes (such as **MHC II** **[G]** or **syndecan** **[G]**) can drastically increase EV production[8,9]. The intracellular trafficking of the recruiting cargoes between plasma membrane and endosomes is also a major regulator of ectosome versus exosome biogenesis[15], although still only modestly appreciated. This complex interdependency between sorting machineries and cargoes needs to be considered carefully as it can lead to the generation of different EV subpopulations with distinct compositions, and hence, potentially different functions.

Regarding cargo carried by these different structures, most EV biologists have mainly focused on membrane cargoes that are exposed on the surface of EVs, because they are strictly associated to membranous structures and more accessible to labelling and analytical approaches. Nevertheless, the EV lumen also contains various amounts of cytosolic material, in particular genetic material (e.g. mRNA, miRNA). The engulfment of small amounts of cytosol and a specific set of cytosolic proteins into ILVs has primarily been described by the autophagy field as the **microautophagy** **[G]** process but its relevance to EV biogenesis is only emerging. The sorting of specific sets of mRNA and miRNA appears to depend on recognition of specific sequence in genetic material by RNA binding proteins (RBP) (reviewed in[16]) and the generation of phase separated condensates of miRNA and RBP into processing bodies **[G]** [17]. Understanding of the sorting mechanisms of RNA and RBPs into EVs are also emerging. They can be passively sorted due to their overabundance in the cytosol, or rely on RBP post-translational modification and interaction with specific sorting machineries such as ESCRT machinery, neutral sphingomyelinase 2 (nSMase2)[18], or autophagosomal marker LC3 on exosomes formed via microautophagy[19], Gag on retroviral-like particles[20] and membrane binding properties[21] on ectosomes. However, discriminating nucleic acids contained within or present on the surface of EVs from nucleic acids simply co-

Commented [MOU5]: Replace by "Gag-like proteins on endogenous retroviral like particles"

isolated with EVs without physical interaction is an issue. This is often due to poorly specific isolation procedures such as high speed pelleting or precipitation that may co-isolate extracellular nucleic acids associated with other extracellular structures (e.g. HDL, protein complexes) [22].

In addition to ectosomes and exosomes, other subpopulations of EVs have been recently described, including migrasomes[23], generated from retraction fibres **[G]** during cell migration; secretory autophagosomes, secretory **amphisomes [G]** [24] and exophers[25] that all likely rely on macroautophagy **[G]**; apoptotic bodies that shed from cells undergoing apoptosis; as well as endogenous retroviral-like particles that bud from neuronal cells. In addition, cells are capable of secreting exomeres, which, however, are non-membranous and should therefore be considered extracellular particles and not EVs.[26]. These different structures expand the known repertoire of extracellular vesicles and particulate matter that is distinct from EVs, and the diversity of mechanisms of their biogenesis (Table 1). In particular, the recent identification of the involvement of autophagy-related processes in EV biogenesis has provided a link between these two fields[27] (Fig. 1a). Macroautophagy processes can intersect with the endosomal pathway[28], notably via the formation of amphisomes through which both ILV and autophagosome content could be released. This leads us to include autophagic mechanisms as new regulators of EV biogenesis and draws attention to the impact of nutrient starvation as a culture condition that would modulate EV biogenesis, and the subpopulations being produced. This is a noteworthy consideration, especially when cells are cultured in the absence of serum, to avoid contamination from particulate material as is commonly practiced in EV studies *in vitro*. The increasing number of biogenesis pathways expands further the diversity of EV subtypes but also offers unique ways to discriminate them via interference with specific mechanisms, thereby providing means to establish a better defined nomenclature and to improve on reporting of the biology of EVs.

[H2] Regulation of EV secretion

Unlike ectosome formation, which by definition takes place at the surface of the cell, exosome secretion requires the highly regulated trafficking of MVEs to the plasma membrane (Fig. 1a). These endosomal compartments need to avoid fusion with lysosomes, to be targeted and to dock and fully fuse with the plasma membrane. It is therefore very challenging to directly compare exosome and ectosome release as they do not follow the same rules.

MVEs have been ascribed to late endosomes whose main fate is fusion with lysosomes leading to degradation of their contents. A still puzzling question is why some MVEs are transported to, and fuse with the plasma membrane. The secretion of exosomes has been associated with impaired lysosomal degradation and could appear as a way for the cell to cope with accumulation of potentially cytotoxic products in the endosomal network. A congested endolysosomal system could then prompt MVEs to find other routes for processing, and secretion may be one such alternative modality[29](Fig. 1a).

MVE refers to a morphological description of an organelle of endosomal origin containing ILVs within its lumen. However, many compartments from early endosomes to endolysosomes and amphisomes contain ILVs and it is not clear which MVE subpopulations are primarily implicated in exosome release as the state of maturation and features of the secretory MVEs are still ill-defined. An interesting concept is that secretory MVEs may correspond to a subpopulation of endosomes[30] that acquire specific molecular regulators such as RAB27 that are associated to lysosome-related organelles[31,32].This implies that secretory MVEs may harbour designated effectors allowing them to traffic toward the cell surface and dock and fuse with the plasma membrane. Acquisition of their fusogenic capacity could rely on the recruitment of specific sorting machineries during ILV biogenesis as ESCRT-dependent and ESCRT-independent pathways have been associated with degradation or secretion of MVEs, respectively[33]. These machineries, by inducing inward

Commented [MOU6]: Add "secretory" lysosome-related organelles

budding of microdomains, could change the composition of the limiting membrane of MVEs and recruit or exclude additional machineries that regulate lysosomal fusion (e.g. TSPAN6), MVE transport (e.g. ORP1L), docking (e.g. RAB27) or fusion (e.g. SNAREs). As membrane cargoes are recruiting the sorting machineries, their accumulation on MVEs or their depletion from endosomes by recycling will also affect these processes and hence exosome secretion. This concept would define exosome secretion as an inducible process that cells engage after stimulation or in pathological situations (tumorigenesis, accumulation of cytotoxic material) and not a constitutive event occurring stochastically or in response to endosomal congestion. The stimuli of EV release are diverse and include physical contact with neighbouring cells to generate ectosomes[34] or provoke exosome release[35], cytokines[36] and calcium[37] that initiates signalling cascades leading to increased exosome secretion (although calcium influx may also affect other secretory pathways such as lysosome secretion).

Generation of ectosomes is supported by various mechanisms acting on plasma membrane microdomains where membrane protrusions act as platforms for their shedding (reviewed in [38]). Ectosomes are either released from tips of membrane extensions such as filipodia **[G]** or **microvilli** **[G]** or by retraction and scission from nanotubes **[G]** and retraction firers such as in the case of migrasomes[23]. Generating EVs from such membrane protrusions could be less energy demanding than from the body of the cell. The molecular regulators of these processes include the actin cytoskeleton and actin-associated regulatory proteins that shape these protrusions. The proteins that organize lipid microdomains such as tetraspanins (tetraspanin-4, CD9), sugar-rich molecules forming the glycocalyx **[G]** and proteins able to bend bilayers such as BAR domain **[G]** proteins are also key regulators of these processes. Biogenesis of ectosomes is modulated by external cues that involve physical (e.g. radiation or adhesion) or chemical factors (e.g. pH)[23,39,40].

The membrane microdomains generating ectosomes are likely not homogenously distributed at the surface of the cell[41]. In addition, MVEs must follow the MTOC (microtubules organizing centres) to release exosomes[35,42]. These spatial constraints raise the question of whether MVE secretion and budding of ectosomes occurs on discrete and defined domains of the plasma membrane. The importance of physical contact with neighbouring cells or with the extracellular matrix, as observed during immune synapse formation[43] or migration[23], support the concept of a polarization of the sites of EV secretion in a highly orchestrated fashion.

By expelling into the extracellular space portions of the cell cytosol and membrane, generation of EVs and in particular large ectosomes raises the question how cells cope with membrane supply and how they balance membrane loss upon secretion of EVs. One way to provide membrane at the site of budding is by vesicular transport; the plasma membrane will provide membrane to the endosomal pathway and vice versa. This suggests that biogenesis of EVs at endosomes and at the plasma membrane are likely connected. This connection is supported by specific machineries that are involved both in EV budding and membrane recycling across intracellular compartments such as syntenin and ARF6 (ref. [9]). Moreover, cargoes that are supposed to recruit budding machineries and drive EV formation continuously cycle between endosomes and plasma membrane (Fig. 1a). A recruiting cargo that recycles from endosomes by retrograde transport or recycling to the plasma membrane would be then depleted from exosome biogenesis sites and likely be enriched in ectosome biogenesis sites. This situation of an interplay between the different subcellular compartments involved in EV biogenesis may hence support a finely tuned balance between exosome and ectosome production. Nevertheless, studies comprehensively addressing regulation of EV secretion in time and space are currently lacking.

[H2] EV biogenesis in the physiological context

Most studies use *in vitro* 2D systems to investigate the biology of EVs. Therefore, we have only a glimpse of how EVs would behave in the complex architecture of a whole organism. There is an urgent need to place investigations of EVs in a more physiologically relevant

context, where heterogeneity of cell types, their spatial arrangement and architecture, together with other potentially relevant microenvironmental parameters such as chemical parameters[44], physical constraints, and other external stimuli[45], are considered. Such consideration is of prime importance for studying EVs that are recovered from patient materials, which can currently only be characterized based on the profiling of cell type specific proteins or specific sorting mechanisms — information insufficient to determine the origin of these EVs and provide means to specifically target signalling routes of these EVs in a clinical setting.

The EV field is now at a point where model organisms and imaging approaches can shed new light on the way that EV secretion can react to physiological constrains[46,47], and much needed new understanding of EV regulation is on the horizon. The investigation of EV biogenesis *in vivo* will notably decipher which EV subtypes follow constitutive mechanisms of generation that cells continuously modulate to adapt their homeostasis such as migrasomes, and which one are secreted in response to specific signals as suggested for exosome release[35]. Answering these questions are of crucial importance as they will notably define EVs as an *in vivo* communication network that provides a true dialogue between cells, and which operates with a considerable degree of context-specificity and precision, rather than providing unspecific signals from bulk clearance of cellular content.

[H1] The dynamics in extracellular space

EVs can be exchanged between cells in an autocrine, paracrine or endocrine manner. Key to the ability of EVs to mediate communication between cells, both locally and at distance, is their capacity to navigate the pericellular and extracellular matrix in which most cells are embedded (Fig. 1b). In some contexts, for example in cancer cell invasion of connective tissue, there is growing evidence of the importance of interactions between EVs and the extracellular matrix[48], but little is known of how these interactions influence EV functionality, or extracellular matrix homeostasis. In healthy tissue, much of our understanding of interactions between EVs and the matrix is informed by the well-established roles of **matrix vesicles** **[G]** (MVs; not to be confused with microvesicles) in the formation of mineralised ECM such as bone[49]. Much less is understood of the mechanisms, or physiological significance, of interactions of EVs with extracellular matrix in soft tissues such as brain or liver, and how this influences ingress to and egress from the circulation and lymphatics for systemic distribution of EVs. In addition, once in the circulation, understanding is limited of EV half-life, of the mechanisms underlying distribution, of uptake of EVs into target tissues from bodily fluids and of tissue tropism. Here, we summarise the current understanding of the influence of EV–extracellular matrix interactions on EV motility and function as well as mechanisms involved in traversing barriers by EVs. We then identify gaps in our understanding of the processes underlying EV distribution *in vivo* (see also Box 2).

[H2] Release of EVs into the extracellular space

Most cells, if not all, are surrounded by a **proteoglycan** **[G]** -rich layer, the pericellular matrix, which separates the plasma membrane and the extracellular matrix[50]. In order to interact with other cells, or enter the lymphatic and circulatory system or other body fluids, EV must first traverse the pericellular matrix, and subsequently the extracellular matrix — in many cases first requiring penetration of a specialized ECM, the basement membrane (Fig. 1b). Intriguingly, a recent report provided evidence that EVs are able to traverse the endothelial basement membrane, but not that of underlying epithelia, suggesting contextual selectivity in the permeability of basement membranes to EVs[51]. Recently, evidence also emerged that EVs released by cells can remain closely associated with the plasma membrane, through tethering proteins such as **tetherin** **[G]** [52], and are also observed throughout the pericellular and extracellular matrix. In the case of mineralised tissues such as bone and dentine, EVs (in the form of matrix vesicles) are a key constituent of the extracellular matrix, acting as mineralisation nuclei and becoming resident in the resulting matrix. Whether EVs have a

synonymous role in soft matrices remains unclear, but extracellular matrix-resident functional EVs have been reported in several tissues (reviewed in [49]). What is well-established, however, is that some EVs are able to navigate and are released from extracellular matrix to enter body fluids. This is enabled by their cargo, including a repertoire of extracellular matrix-modifying proteins, such as matrix metalloproteinases (MMPs), matrix receptors such as integrins, and enzymes involved in matrix cross-linking such as **lysyl oxidase** **[G]** (LOX) and **transglutaminase** **[G]** (TG)[53]. Although the roles of these different matrix modifying proteins in EV motility have yet to be fully elucidated, evidence from studies of cancer cell migration indicate that the release of EVs by **invadopodia** **[G]** at the leading edge of cancer cells may drive directionality of invasion, and promote cellular migration through the matrix, and that this behaviour is modified by the physical properties of the extracellular matrix[54].

[H2] Traversing biological barriers

Having traversed the ECM, a proportion of EVs released by cells enter the circulatory system and **integumentary system** **[G].** The first hurdle encountered at this stage of the EV journey is crossing the barrier formed by endothelial cells lining blood and lymphatic vessels. Much of what we understand about the mechanism of intravasation by EV is gleaned from studies of EV interactions with the blood brain barrier (BBB), a specialised endothelial layer. Several lines of evidence indicate EVs are able to cross the BBB by transcytosis (Fig. 1b)[55]. The mechanisms by which EVs escape from the recipient endothelial cells remain opaque, but are suggested to be via the formation of intraluminal vesicles and subsequent release from the cell. It remains to be determined whether these mechanisms hold true more widely for EVs traversing endothelial barriers. To date, no evidence has been found for a para-cellular route of transport in healthy tissue, but a recent study[56] reported the ability of HeLa cell-derived EVs to degrade endothelial cell junctions, disrupting barrier integrity and – it could be hypothesised – enabling the entry of cancer cell-derived EVs into the circulation. In addition, a recent report provided evidence that EVs released by cancer cells depleted of adhesion molecules RalA and RalB were less able to promote endothelial permeability compared to their wild-type counterparts, suggesting that the spreading of EVs is specifically regulated by EV composition[57].

[H2] EV distribution in vivo

On entering the vasculature or lymphatics, a number of questions remain unanswered about the fate of EVs. In cancer, several lines of evidence indicate the ability of EVs to exhibit organotropism, homing to particular tissue environments — both locally and at distance — dictated by receptors and other factors present on the EVs, and signals derived from recipient cells. An elegant series of experiments in zebrafish embryo model demonstrated that melanoma-derived EVs were taken up by endothelial cells and macrophages[58]. In macrophages, endocytosis of EVs resulted in a phenotypic switch, indicative of the ability of EVs trafficked via the circulatory system to influence the behaviour of distant cells. A report published concurrently demonstrated the release of large quantities of endogenously-derived EVs into the circulation from the yolk-sac during zebrafish embryogenesis[59]. These showed similar characteristics to EVs derived from melanoma cells, being endocytosed by endothelial cells and macrophages predominantly, suggesting this mechanism also occurs in normal physiology. It is important to note that endothelial cells will be among the first encountered by EVs released into the circulation, and this, alongside the scavenging role of macrophages, should be taken into account before inferring specificity of EV uptake. In the fly, microvilli-derived EVs were recently detected in the extracellular space and reported to play an important role in long-range Hedgehog signalling[47].

Data from humans and other mammals regarding the fate of endogenously-derived EVs in either the circulation or lymphatics remains limited, and caution must be exercised in extrapolating findings in model organisms to humans. In rats, the generation of a transgenic line endogenously expressing GFP-labelled CD63 (an ILV/exosome-enriched molecule) demonstrated the presence of endogenously-generated fluorescently-labelled EVs in several

body fluids, and *ex vivo* demonstration that these could be taken up by fibroblasts in culture, but the precise cell-type of origin and fate of these EVs *in vivo* could not be mapped[60]. Another study in mice used a Cre recombination approach to determine that functional EV cargo derived from cancer cells only acted at a local level, that is within the tumour microenvironment[61]. Support for primarily local transmission of EVs was also provided by demonstrating that most tumour-derived EVs disseminate to the local lymph nodes where, at least in early disease, subcapsular sinus macrophages prevent wider transmission by internalizing them and thereby providing a sink for EV clearance[62]. Besides local transmission, it has been suggested that systemic, functional transfer of cargo — specifically mRNA — via cancer-derived EVs may occur[63].

Several other studies have assessed the biodistribution of exogenously introduced EVs in animal models. This results in a mixed picture, with the majority of studies indicating preferential accumulation in the liver and spleen, but others demonstrating tropism to other tissues, and providing some indication of context specific distribution; in several cases the distribution of EVs differed depending on the cellular source of the EVs[64]. Many of these studies employed cancer cell-derived EVs, for which there is growing evidence of some selectivity of tissue targeting; there remains, however, much for us to learn of the determinants of EV biodistribution, with important consequences particularly for attempts to use EVs as drug-delivery vehicles[65].

Current comprehension of the dynamics of EVs in the extracellular space (~~Fig.1, steps 7-10 ;~~ Fig.1, steps 7-10 ~~?~~; Fig. 2, steps 1-4) comes from studies *in vitro*, or using exogenously administered EVs *in vivo*. The relevance of such studies to endogenous, physiological EV-mediated crosstalk can be questioned. Thus, in depth studies investigating the fate of endogenous EVs are warranted, but require novel experimental models and these come with different challenges. A standardised, multimodal approach is urgently required to better understand the EV journey from the cell of origin to its final destination, be it a specific recipient cell or a site of clearance.

[H1] Functional delivery of EV cargo

The uptake of EVs by different cell types is a well-observed phenomenon but the processes directing EV entry into recipient cells remain poorly understood. EV acquisition may be considered as a series of steps (Fig. 2, steps 5-9). The initial encounter with a recipient cell involves physical association directly through a variety of receptors and/or bridging molecules at the plasma membrane (Fig. 2, step 5). This binding is recognized, and drives intracellular signalling events establishing finally the process of cell entry, as well as other downstream effects on the cell. Whilst the routes of internalisation (Fig. 2, step 7) are dominated by endocytic processes, other modalities such as direct membrane fusion with the recipient plasma membrane, at least for some vesicle types may occur, although extensive evidence of fusion as a general mode for EV operation is rather scarce. There are also some examples of EVs retaining a fairly stable plasma membrane localisation after encounter with the recipient cell, apparently not gaining entry (Fig. 2, step 6). This, however, does not exclude their functionality. One example here are B cell-derived EVs coating follicular dendritic cells, and thus conferring antigen-presenting function through receipt of MHC Class-II[66]. The generally accepted paradigm is that functional effects on recipient cells, besides direct signalling from the plasma membrane, may require EV internalisation followed by cargo release into the cell (Fig. 2, step 8). The intracellular handling of EVs certainly involves degradative processes, whereby internalized EVs are targeted to lysosomes (Fig. 2, step 9). Overall, so far little is known about intracellular processing of EVs and how delivered instructions are interpreted by the recipient cell and several questions remain to be addressed in these areas to better understand the functional impact of EV-mediated cell–cell communication (Box 3).

[H2] EV interaction with the cell surface

In terms of EV interaction with the cell surface, the molecular complexity at the outer EV surface, involving proteins, lipids and glycosylation, is challenging to decipher, and is likely diverse across distinct subsets of EVs[67]. These features are also dynamic with changes imposed by donor cell status and the nature of the pericellular microenvironment. An attractive paradigm of EV-mediated cell–cell communication would rely on the existence of an 'address system', where the EV membrane composition provides a high degree of selectivity in terms of targeting specific recipient cell types. There are notable examples *in vivo*, where EVs with distinct composition are able to target specific organs to elicit microenvironment changes in a remarkably selective fashion[68]. However, there are also extensive data pointing towards recipient cell promiscuity, with poorly cell-selective mechanisms of interactions. **Phosphatidylserine** **[G]** (PS) exposed on EV outer membranes is a major example of general, non-selective targeting, with PS-mediated interactions involving bridging molecules (MFG-E8, Protein S, GAS6 and others) or a range of PS receptors that mediate cell attachment and entry. Surface exposed PS is a well-documented feature of apoptotic bodies, whereby the detection of PS promotes their engulfment, and clearance, by macrophages. Hence this mechanism may point to macrophages as a preferential recipient for EVs, but the repertoire of cell types capable of binding and taking up EVs is indeed much broader than this.

Many other modes of EV-attachment to recipient cells have been described, detailing roles for tetraspanins, heparan sulphate proteoglycans, integrin receptors, tetherin, and many other elements[52,69,70]. For any given vesicle population therefore, there may be several different interaction elements that could target the EV to a number of cell types. Deciphering the hierarchy of importance for EV attachment to the target-cell surface, signalling from the cell surface and cell entry is an enormous challenge. It is important to acknowledge also that some of the EV surface components may play negative, regulatory functions during cell interactions such as CD47 which inhibits phagocytic uptake[71] [72]. Hence, ultimately, the outcomes of EV interactions with target cells will depend on the net balance of positive and negative influences.

In addition to the constituents of EVs that are endogenously provided by the donor cell, there is an emerging recognition of the relevance of the protein corona[73]. This is a coating of the EV surface with factors present within the peri-vesicular environment that is acquired after EV release, and provides additional nuance, complexity and dynamics in altering the composition of EV surfaces. For example, deposition of clotting factors, **complement** **[G]** , immunoglobulins, soluble growth factors, chemokines or matrix constituents onto the EV surface can influence interactions and responses driven by EVs[74].

[H2] Routes of cell entry

Endocytosis is a key mechanism driving EV entry into cells. Mammalian cells depend on a diversity of endocytic mechanisms that occur concurrently, including clathrin-dependent and clathrin–independent mechanisms as well as **macropinocytosis** **[G].** Specialized phagocytic cells are furthermore capable of ingesting other cells and/or particles via phagocytosis. Interestingly, all of these different endocytic mechanisms have been implicated in EV internalization, which is likely a result of the large diversity of EV donor and recipient cell pairs that have been reported, as reviewed in [5]. The mechanism of internalization may be relevant to the fate of EVs and their cargo. For example, when studying EV-mediated small RNA delivery, a poor correlation between EV/small RNA uptake efficiency and functional small RNA transfer (assessed via target gene knockdown) was observed among different cell types, including phagocytic Kupffer cells[75]. It is thus tempting to speculate that different EV internalization mechanisms may lead to different functional outcomes: degradation versus functional transfer of EV cargo.

[H2] Intracellular trafficking and signalling

In general, endocytosis in mammalian cells leads to internalization of cargo from the plasma membrane, and transport from early endosomes to either recycling endosomes through which the cargo is recycled back to the plasma membrane, or to late endosomes and finally lysosomes where it is degraded. EVs seem to follow a similar path, with evidence suggesting that EVs, or at least the dyes or labels that are used to visualize them, eventually end up in lysosomes[69,76]. Accumulation of EVs in lysosomes may also reflect the final destination of EVs that have signalled at the surface of the recipient cells. While lysosomes are often seen as sites for breaking down biomolecules (and therefore considered non-functional EV destinations), increasing evidence suggests they also function in various cellular processes such as plasma membrane homeostasis, signalling and energy metabolism. Indeed, lysosomal catabolism of EVs by endothelial cells has been suggested to provide these cells with trophic support[59]. Thus, lysosomes may serve as a site of functionality for EVs, and may not be simply a compartment for their destruction.

At the same time, there is overwhelming evidence that EVs are capable of transferring their luminal cargo, including RNA, into the cytosol of recipient cells, which indicates that EVs are capable of escaping the endolysosomal pathway. How EVs induce such endosomal escape is largely unknown, however there is some evidence that this occurs through EVs fusing with late endosomal membranes[77,78], potentially triggered by acidification along the endolysosomal axis[79,80]. Whether there are intrinsic differences in EV subtypes which are destined for lysosomal degradation or cargo release through fusion, or whether EV fate is dictated by the characteristics of the recipient cell remains unknown.

[H2] Cell–cell communication via EVs

Overall, the key challenge to a better understanding of the cell-cell communication networks that are mediated via EVs is to correlate the molecular mechanisms underlying EV targeting, internalization and trafficking to EV function and its physiological implications (Fig. 3). Solving this challenge will likely require an integrative approach in which a physiologically relevant set of EV donor and acceptor cells is studied in a multicellular and three-dimensional environment mimicking the desired in vivo context, using a combination of ~~(super-resolution)~~different microscopy techiques and complementary approaches to track single EVs and their cargo. This also requires functional readouts that can be directly linked to EV signalling or cargo transfer. Such an approach awaits both technological advances as well as an increased understanding of basic EV biology as outlined in the previous sections.

Commented [PS7]: Au: What is meant by: 'a combination of (super-resolution) microscopy'? Do you mean a combination of different microscopy techniques or a combination with yet another technique?

[H1] Future directions

Despite the enormous progress that has been made over the past years, working with EVs still presents several challenges that are shared by many colleagues in the field (Fig. 3). These include a lack of appropriate methodology, insufficient reporting of important details in published works that limit reproducibility, disagreements over nomenclature, a need for more standards and controls, and technical difficulties stemming from the inherent properties of EVs. However, through concerted efforts these challenges are being slowly met.

[H2] Technical challenges

Simply put, we do not yet have the toolset we need to properly study EVs. That is not to say that the tools that have been developed thus far are not of value; they have in fact been crucial in helping us to reach a good level of understanding, and they continue to yield new insight into how EVs function. The biology of EVs, however, presents a particular challenge to the unwary researcher, and in particular the heterogeneity of EV preparations and low levels of material requires new sensitive and nanoscalar methodologies and approaches[81].

Several commonly used EV purification methods exist, but all have limitations, producing EVs with different yields and purity [81,82]. Choosing the most appropriate technique is therefore a trade-off of these different parameters, with the final choice also depending on the downstream application of the EVs[82]. A common drawback to all the bulk-techniques that separate on the basis of physical parameters, including size or density, is that they yield heterogeneous subpopulations of EVs[83]. Methods that rely on affinity-based capture of EVs with specific surface markers can yield relatively 'purer' populations, but to date there are no definitive markers that can specifically identify a single subpopulation. Caution is therefore required when interpreting the analysis of such EV populations, and we advocate for the development of novel techniques for the rapid isolation of pure EV subpopulations.

The relative paucity of material of EVs also presents a technical issue. Most current analysis methods rely on generating sufficient material by aggregating large numbers of EVs. These 'bulk' characterisation methods only report the average state of the EV population, and do not permit easy access to understanding the heterogeneity of EV preparations[81]. Single EV methodology is gaining traction in the field and can be used to quantify EVs or analyse their composition[84]. Techniques such as Nanoparticle tracking analysis and Tunable Resistive Pulse Sensing can be used to estimate particle size and numbers, while emerging techniques such as Imaging Flow Cytometry[85], Nano-flow Cytometry[86], and Single Particle Interferometric Reflectance Imaging Sensor technology[87] are able to characterise EV size and content of more than one fluorescently marked component, providing a window for analysing heterogeneity. An important caveat is that both the measured size distribution and concentration are determined by the instrument's detection limit, which should be taken into account when interpreting results. Such details about single EVs allow us to potentially sort selected subpopulations from the bulk EV population, and hence ascribe a more detailed mechanistic description of their function. – Currently, the complexity of the bulk population presents major challenges when trying to define molecular basis for vesicle-mediated effects

The size of many EVs provides the field with another technical barrier. The most intensively studied EVs are in the 30-150 nm range, which is below the limit of diffraction for ordinary light microscopes. This can make imaging the release, transit and uptake of EVs challenging. The use of super resolution microscopy techniques can help to resolve EV structures and interactions, but increased spatial resolution often comes at the cost of temporal resolution, making the resolution of EVs in live cells a challenging goal. The use of electron microscopy can give nanometre resolution, but must be done on fixed samples. Here, the use of correlative light and electron microscopy (CLEM) can bridge the gap between multi-colour

fluorescent microscopy in live or fixed cells and ultrastructural studies of EVs[36]. Even more challenging, yet of crucial importance is to characterize their endogenous behaviour, without imposing the varied biases of isolation. To achieve this, such as by *in vivo* imaging of EVs is a goal, but currently limited to model organisms such as *D. rerio* (zebrafish) and *D. melanogaster*[46].

With many of these light microscopy methods, EVs must be labelled to make them visible to the microscope[88]. There are a variety of labelling methods that have different strengths and weaknesses[46]. Resolving these limitations remains an important goal to move the EV field forward.

[H2] Standardization of research

The exponential increase in publications and interest in EVs help to drive the field forwards, but also bring new difficulties. The proliferation of terminology and methodology employed in studies across the world leads to more variability, and incomplete reporting can make interpreting and comparing different studies more challenging. The existence and continuing discovery of EV subtypes adds further complexity to this issue[8,14,22,26]. To address this, members of the International Society for Extracellular Vesicles (ISEV) have attempted to foster some degree of standardisation and agreement in the nomenclature, by proposing that the naming of the different types of EVs should be based on biogenesis pathways, and that they should be collectively termed EVs[89]. The EV-TRACK initiative, which aims to catalogue the methodology of publications that include EV-related experiments, has revealed that hundreds of different protocols have been used, even when the fundamental type of purification method was the same, and that many studies did not report the methodology in sufficient detail for others to reproduce the experiments[90]. This highlights the need for improved reporting of those in the community. Finally, ISEV members have introduced the concept of the 'Minimal Information for Studies of Extracellular Vesicles' (MISEV), a framework to assist researchers on the experimental requirements and reporting guidelines for publications on EVs[6]. These MISEV guidelines are not intended to be a rigid set of rules, but are intended to be applied with common sense, and their aim, ultimately, is not to stifle pioneering research, but to raise the standard of publications across the field and help to educate researchers that are new to the field.

[H2] Applications and translational opportunities

This Expert Recommendation has focused on the basic biology of EVs, and in particular their biogenesis, transit and uptake by target cells. Nevertheless, better understanding in these areas can lead to numerous applications and translational opportunities. EVs have been implicated in numerous biological processes, and their biogenesis and functions can become corrupted in various diseases. The degree to which EVs directly contribute to physiology and pathology remains to be determined, but a growing body of evidence shows that they can and do play a role in the development and evolution of at least some disorders, including cancer.

EVs are found in every body fluid, potentially providing a window into the physiological (or pathological) state of the cells that are contributing to the extracellular milieu. The cellular changes that occur in pathological conditions, either as a cause or effect of the disease, can be reflected in the content of EVs that are released by such cells. EVs with altered contents can be identified with *a priori* knowledge, such as through detection of specific cargoes (including nucleic acids or proteins), or *a posteriori* through screening and omics approaches. A better understanding of how EVs are released by cells, how they transit to specific body fluids, and how their steady state levels change in circulation in various physiological contexts and under different disease conditions would lead to more robust biomarkers for tracking disease onset and progress.

The ineffective delivery of therapeutic cargo is a key obstacle for the treatment of many diseases. EVs are naturally occurring vehicles that are capable of transferring biological cargo, and as such there has been a great deal of interest in their potential applications in drug delivery[91] (Table 2). In order to fully capitalise on their potential, we need a better understanding of the rules that govern the normal EV journey in vivo. This includes knowledge on how EVs interact with different matrices and are maintained in (or cleared from) biological fluids, how they cross biological barriers, how tropism to specific tissues is determined, how they are taken up into cells and how cargo is functionally delivered. Whilst a great deal of clinical validation is needed for the regulatory approval needed for EV-based diagnostics and therapeutics, the incremental gains achieved through the study of basic EV biology will eventually unlock the full range of EV applications.

[H1] Conclusion

The EV field has made tremendous leaps forward in the past two decades. This is evident from the increase in high-quality publications, and development of techniques appropriate for the study of these small messengers. Through the collaborative publication of guidelines and agreement on standards and nomenclature, the research in the field continues to evolve into a more cohesive approach. This has led to advancements in many areas of EV biology, particularly in EV biogenesis and, to some extent, EV uptake. Knowledge of EV transit *in vivo* and the mechanisms of cargo delivery lag behind, and are areas that we recommend requires more attention. Achieving this will require further investment from funding bodies and the development of more tools and *in vivo* models to study EVs. We particularly encourage the interaction of researchers from different disciplines, including basic cell biologists, clinicians, technology specialists as well as computer scientists, as truly inter-disciplinary cross-pollination can lead to quantum leaps in any given field. Without a doubt, this exciting field is progressing rapidly, which will continue to have an impact on our fundamental understanding of how multicellular organisms are regulated.

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Competing interests

DRFC is an employee and shareholder of Evox Therapeutics. PV serves on the scientific advisory board of Evox Therapeutics. Remaining authors declare no competing interests.

Author contributions

The authors contributed equally to all aspects of the article. ~~OR All authors/X.X. researched data for the article. All authors/X.X. contributed substantially to discussion of the content. All authors/X.X. wrote the article. All authors/X.X. reviewed and/or edited the manuscript before submission.~~GvN designed the figures.

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Related links

EV-TRACK: https://www.evtrack.org/

Figure legends

Figure 1: State of the art of extracellular vesicle biogenesis and dissemination in the extracellular space. a) **(Steps 1-6)** Two main extacellular vesicle (EV) subtypes include ectosomes and exosomes. Ectosomes bud off directly from the plasma membrane (1), whereas the release of exosomes involves the formation of multivesicular endosomes (MVEs) that upon transport towards and fusion with the plasma membrane release their intraluminal vesicles (ILVs) as exosomes (2). Endocytosis and endocytic recycling of potential cargoes regulate the generation of these EVs (3). Various sorting mechanisms (ESCRT-dependent and ESCRT-independent) linked to the post-translational modification of cargoes induce the budding and fission of membrane microdomains enriched in specific sets of proteins, lipids and nucleic acids (4). Release of exosomes requires extra steps to prevent targeting of MVEs for lysosomal degradation (5). The process of EV release can also intersect with autophagy (6), including the role of microautophagy in ILV generation and formation of secretory amphisomes upon fusion of autophagosomes and MVEs. These can release heterogenous content into the extracellular space, including secretion of ILVs and autophagosomal markers and digestion products. In addition, mature autophagosomes can also fuse with the plasma membrane generating secretory autophagosomes. These mechanisms expand the variety of EV subpopulations and shows that EV generation involves multiple pathways. Dashed lines indicate processes that present alternate fates for ILVs other than their exocytosis as exosomes. **b)** **(Steps 7-10)** Once released, EVs may directly interact with pericellular and extracellular matrix that will influence their dissemination (7). EVs can be directly released into biological fluids (8) or reach them by transcytosis or by passing through breaches of biological barriers (9) to disseminate through the organism via the circulation (10).

Figure 2: The dynamics of extracellular vesicles in extracellular space leading to their uptake and functions in recipient cells. Extracellular vesicles (EVs) can reach recipient cells in an autocrine, paracrine or endocrine manner (1). EVs (or their specific subpopulations) likely face substantial clearance by macrophages or end up in a detoxifying organ such as the liver (2). Nevertheless, through mechanisms that remain to be fully determined, EVs can cross biological barriers to reach recipient cells (3). Interaction of EVs with the extracellular and pericellular matrix may further affect their progress towards recipient cells through potentially selective binding interactions (4). Specific engagement of recipient cell receptors with EV-associated ligands will likely define preferential tropism of EVs for certain cell types. At the recipient cell level, specific engagement of EVs with receptors can lead to activation of signalling cascades (5), decorating the recipient cell surface, resulting in the conferral of new functions (6), or their uptake by various mechanisms (7). EVs have the capacity to deliver or transfer their surface and intraluminal content via still ill-defined but likely fusogenic processes into the cytosol, where these molecules can perform specific functions. This occurs most likely once EVs have been internalized, although direct fusion of EVs with the recipient cell has also been suggested (8). Constituents remaining within endocytic compartments for the most part end up in degradative compartments where their catabolism could provide trophic support to the recipient cell (9).

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Figure 3: Key questions and challenges in studying extracellular vesicle biology. Schematic representation of the journey of extracellular vesicles (EVs) and other means of intercellular communication between a producing and a receiving cell by autocrine, paracrine and endocrine routes. The coloured boxes highlight key question and main challenges for the field.

Table 1. Subtypes of extracellular vesicles/particles characterized to date

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	Size	**Markers**	**Biogenesis / release**	**References**
Exosomes	30-150 nm	ESCRT complex proteins, CD63	Formed as intraluminal vesicles (ILVs) involving inward budding of the endosomal membrane via ESCRT-dependent or ESCRT-independent mechanisms of cargo sorting and/or via microautophagy. Released upon fusion of multivesicular endosomes (MVEs) with the plasma membrane	[8,9,12,31]
Ectosomes (including microvesicles and oncosomes)	50-10000 nm	Annexin A1, ARF6	Outward budding of the plasma membrane, scission/pinching off from membrane protrusions	[15,92,93]
Migrasomes	500 – 3000 nm	TSPAN4	Generated from retraction fibres and released during cell migration	[23]
Secretory autophagosomes/Amphisomes (2)	Not determined	LC3	Generated through macroautophagy (secretory autophagosomes) or fusion of autophagosomes and MVEs (amphisomes). Fusion with the plasma membrane leads to release of single membrane-bound autophagosomes or for amphisomes, their internal content including autophagic content and ILVs.	[27,28]
Exomeres*	< 50 nm	Unknown	Unknown	[26,94]
Retroviral-like particles	Not determined	Gag-like proteins (Arc1, Arc2)	Unknown	[20,95]

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Exophers	1000-10000 nm	Phosphatidylserine, LC3, Tom20	Unknown (but likely related to macroautophagy)	[96]
Apoptotic bodies	50-5000 nm	Phosphatidylserine	Released from apoptotic cells upon activation of apoptosis related transduction pathways	[97]

This table provides the reader with findings from selected reports. One should note that the biogenesis/release mechanisms and cargo may differ between cell types. * In contrast to other species in this Table, exomeres are not membrane-bound and are not classified as extracellular vesicles

Table 2: Clinical trials with extracellular vesicles as therapeutic delivery systems

NCT number	Phase	Status	Condition	Extracellular vesicle source	Therapeutic	Reference
NCT04592484	Phase 1/2	Recruiting	Advanced Solid Tumours	HEK293	STING agonist	[98]
NCT03608631	Phase 1	Recruiting	Metastatic Pancreatic Adenocarcinoma; Pancreatic Ductal Adenocarcinoma	Mesenchymal Stromal Cells	KRAS G12D siRNA	[71,99]
NCT01294072	Phase 1	Recruiting	Colon Cancer	Plant	Curcumin	Not available
NCT03384433	Phase 1/2	Recruiting	Cerebrovascular Disorders	Mesenchymal Stem Cells	miR-124	Not available
NCT01854866	Phase 2	Unknown	Malignant Pleural Effusion, Malignant Ascites	Autologous Tumour Cells	Chemotherapeutic drugs	[100]
NCT02657460	Phase 2	Unknown	Malignant Pleural Effusion	Autologous Tumour Cells	Methotrexate	[101]

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Box 1: Extracellular vesicle biogenesis: Key questions and challenges
- How do we take into account the whole complexity of the cell, including regulation of intracellular trafficking and cell metabolism in the study of extracellular vesicle (EV) biogenesis? - Since EV biogenesis is also dependent on interaction with neighbouring cells and matrix *in vivo*, to which extent does the "EV secretome" change depending on environmental cues? - Are distinct subsets of exosomes with different functions associated with multivesicular endosome (MVE) subpopulations? - What are the processes that turn subpopulations of MVEs into secretory organelles? - How do chemical parameters such as pericellular pH, concentration of reactive oxygen species, and osmotic pressure, as well as physical constrains of the tissue such as mechanical pressure due to cell density or stiffening extracellular matrix affect ILV generation and secretion and plasma membrane budding? - Are processes described *in vitro* relevant *in vivo*? - From a more practical perspective, when aiming to interfere with EV biogenesis, how can we take into account the complexity and diversity of the biogenesis of subpopulations of EVs, the cell types, the culture conditions and the level of expression of EV cargoes? For instance, a given pathway such as **ceramide** **[G]** production may inhibit exosome secretion of one subtype but not others, while simultaneously affecting other regulatory processes in the cell. Moreover, if the inhibition or impairment of a specific process affects MVE biogenesis, given not all MVEs are secretory, new approaches are needed to clearly distinguish correlation and causality of the importance of the given process during exosome secretion.

Box 2: The dynamics of extracellular vesicles in extracellular space: Key questions and challenges
- What is the interrelationship between extracellular vesicles (EVs) and the extracellular matrix? - Are all EVs capable of crossing all basement membranes, and, if not, is there tissue/subpopulation specificity? - Given the importance of the extracellular matrix in the maintenance of normal tissue function, how do mechanical forces and the local matrix composition influence EV–extracellular matrix interaction? - To what extent does the composition of the extracellular matrix modulate the composition and hence function of EVs? - What types of EVs interact with the extracellular matrix, and is release of specific subpopulations into the extracellular matrix directly dependent on plasma membrane–matrix interactions and associated mechanical forces? - Do EVs carry components/properties that allow them to resist degradation extracellularly, and to avoid undesirable interactions with non-target cells, matrix constituents and other interstitial factors? - How important are the biophysical properties of EVs (e.g. their size and compressibility) in traversing through the complex extracellular microenvironment? - After traversing the extracellular matrix, EVs, or at least a subpopulation thereof, appear to have the capacity to cross several biological barriers. Is this capability influenced by specific pathological situations (e.g. the increase in vascular permeability)? - Do specific cell types interact with EVs more efficiently, and does this interaction differ between different EV subpopulations? - Most of our understanding of the fate of EVs *in vivo* comes from studies using exogenously administered EVs. However, EV isolation, storage, method of formulation and labelling may affect their targeting specificity and ultimate fate. So, are the results obtained using exogenously administered EVs representative of the physiological fate of endogenously generated EVs? - Multiple approaches have been used to assess the biodistribution of EVs *in vivo*, including fluorescent labelling of lipids and proteins, immunofluorescence, bioluminescence, PET, SPECT and MRI and CT imaging[59]. All of these approaches have limitations in tracking the fate of EVs, and novel approaches with large dynamic ranges of both temporal and spatial resolution are required to overcome these.

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Box 3: Uptake and functional delivery of extracellular vesicle cargo: Key questions and challenges
- Which extracellular vesicle (EV)-associated factors are the critical ones for dictating function (defined by signal-induction and/or cell entry)? - Can we define the rules that govern EV-targeting, and can we manipulate this knowledge in future translational studies using EV based-medicines? - To what extent is the nature of the protein corona controlled by the native EV composition and how important is the corona in determining interactions with recipient cells or whole systems? - How do we overcome the technical challenge of small EV size and scarcity of cargo [, posing issues on direct imaging of functional delivery of cargo by native EVs *in vivo*? - Are different EV subpopulations processed differently in recipient cells and do they exert different cellular functions? Can this heterogeneity be addressed by studying EV subpopulations individually, or do they cooperate in a synergistic manner? - How do we provide direct evidence that observed physiological effects are truly EV-mediated and if so, what EV cargo(es) is/are responsible? In this regard, an overlooked aspect is the relative contribution of EVs compared to other communication means such as cell–cell contact, nanotubes or soluble factors such as cytokines or extracellular RNA. - When attempting to dissect the relative potency of EVs in directing cell communication from other secretome constituents, how important are the different non-vesicular secreted factors, such as extracellular matrix proteins and cytokines in their co-operation with EVs in dictating the final and complex cellular response?

Glossary terms

Oncosomes – Large extracellular vesicles generated from the plasma membrane of cancer cells.

ESCRT machinery – Protein machinery composed of several multiprotein subcomplexes that enable membrane remodelling at endosomes, plasma membrane or nuclear envelope resulting in membrane budding.

Syntenin-Alix pathway – Alternative sorting mechanism at endosomes to generate intraluminal vesicles that shortcuts the first subunits of the ESCRT machinery.

Tetraspanins – Family of transmembrane proteins that are enriched in various subtypes of EVs and are characterized by their capacity to associate into dynamic membrane microdomains.

Arrestin domain-containing protein 1 – Protein adaptor for the Nedd4 family of ubiquitin ligases that is involved in the generation of EVs at the plasma membrane.

MHC - Transmembrane protein heterocomplex expressed by antigen presenting cells that present antigenic peptides to T-cell receptors.

Syndecan – Single transmembrane domain protein that is thought to act as coreceptor, especially for G protein-coupled receptors, and is known to engage the syntenin-Alix pathway for sorting to exosomes.

Microautophagy – Sorting process occurring at the late endosome or lysosome to engulf cytoplasm and cytosolic proteins into intraluminal vesicles.

Processing bodies –Distinct foci consisting of many enzymes and nucleic acids, formed by phase separation within the cytoplasm of the eukaryotic cell and primarily involved in mRNA turnover

Retraction fibres – Membrane elongated structures generated at the rear of migratory cells connecting the adhesion pattern to the round cell body.

Amphisomes – Chimeric organelle resulting from the fusion of autophagosomes and multivesicular endosomes.

Macroautophagy – Intracellular process leading to the specific enwrapping of cytosolic material and organelles by membranes to target them to lysosomes for degradation.

Filopodia – Cytoplasmic projections that extend beyond the leading edge of migrating cells
Microvilli – Membrane protrusions, primarily generated in epithelia, involved in absorption, secretion and adhesion.

Nanotubes – Membrane elongated structures connecting two cells

Glycocalyx – Set of glycolipids and glycoproteins present on the extracellular surface

BAR domain – Highly conserved protein dimerization domain displaying a banana shape that preferentially binds to curved membranes and sustains membrane deformation and traffic.

Matrix vesicles – Extracellular spherical bodies selectively located in the pre-mineralized matrix of cartilage, bone, and dentin.

Proteoglycan – A family of ubiquitous, heavily glycosylated proteins that function as critical components of the extracellular matrix.

Tetherin – Lipid raft associated integral membrane protein that tethers virus-like particles and exosomes thereby inhibiting them from discharging into the extracellular milieu.

Lysyl oxidase – Enzyme that induces crosslinking of extracellular matrix proteins by converting lysine molecules into highly reactive aldehydes.

Transglutaminase – Enzyme that induces crosslinking of extracellular matrix proteins by generating isopeptide bonds.

Invadopodia – Specialized actin-rich membrane protrusions that concentrate high proteolytic activities and are capable of crossing extracellular barriers.

Integumentary system – Organ system forming the outermost layer of an animal's body and includes skin, hair, nails and exocrine glands.

Phosphatidylserine – Phospholipid commonly found in the inner leaflet of biological membranes, which gets exposed on the surface of apoptotic cells and is used by viruses and extracellular vesicles to enter cells via apoptotic mimicry.

Complement – System of plasma proteins that upon activation leads to opsonisation and engulfment of pathogens as part of the innate immune system.

Macropinocytosis – Regulated form of endocytosis that involves non-selective uptake of extracellular material.

Ceramide – Sphingolipid that induces inward budding of endosomes to produce intraluminal vesicles in an ESCRT-independent manner.

eTOC

Extracellular vesicles (EVs) have emerged as means of cell–cell communication, with documented roles in both physiological and pathological conditions. Still, many questions remain to be answered about the mechanisms governing functional delivery of EVs between donor and recipient cells and the importance of this communication in vivo.