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The Challenges and Possibilities of Extracellular Vesicles as Therapeutic Vehicles

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ABSTRACT

Extracellular vesicles (EVs) are small lipid-enclosed particles that can carry various types of cargo, including proteins, nucleic acids and metabolites. They are known to be released by all cell types and can be taken up by other cells, leading to the transfer of the cargo they carry. As such, they represent an important type of intercellular signalling and a natural mechanism for transferring macromolecules between cells. This ability to transfer cargo could be harnessed to deliver therapeutic molecules. Indeed, a growing body of work has described the attempt by the field to utilise EVs to deliver a range of therapeutics including RNAi, CRISPR/Cas9 and chemotherapeutics, to a specific target tissue. However, there are numerous barriers associated with the use of EVs as therapeutic vehicles, including the challenge of efficiently loading therapeutics into EVs, avoiding clearance of the EVs from circulation, targeting the correct tissue type and the inefficiency of internalisation and functional delivery of the cargo. Despite these difficulties, EVs represent a tremendous therapeutic opportunity, both for the delivery of exogenous cargo, as well as the therapeutic benefit of targeting aberrant EV signalling or treating patients with natural EVs, such as those released by mesenchymal stem cells. This review describes current knowledge on the therapeutic potential of EVs and the challenges faced by the field. Many of these challenges are due to a lack of complete understanding of EV function, but further research in this area should continue to yield new solutions that will lead to the use of EVs in the clinic.

1. Introduction

Extracellular vesicles (EVs) are emerging as invaluable tools for the delivery of therapeutics to cells. EVs are endogenously released from nearly all reported cell types and are used to communicate to nearby or distant cells [1]. They can act as signalling devices in themselves, presenting antigens on the EV surface to cells, binding to receptors and providing trophic support [2,3]. They can also successfully deliver functional cargo to recipient cells. EV cargo is encapsulated within a lipid membrane and is composed of a combination of proteins, carbohydrates and various nucleic acids including DNA, mRNA and non-coding RNA native to the donor cell. EV cargo transfer can result in induction of cell signalling [4], *de novo* gene expression, posttranslational modification or translation of new transcripts depending on what cargo is successfully delivered to the recipient cell [5].

There are three main classifications of EVs based on their biogenesis (as reviewed in [2,6]); exosomes, which are formed as intraluminal vesicles in multivesicular bodies (MVB) and released when this fuses with the plasma membrane, microvesicles, which bud off the plasma membrane, and apoptotic bodies, which are released by cells undergoing apoptosis. Typically, EVs sizes range from 30 - 1000 nm. EVs are involved in a wide range of biological processes from development, immune modulation, to evolving drug resistance [7–11]. EVs have been heavily implicated in cancer, amongst other pathologies, and have roles in every stage of the metastatic cascade (reviewed in [12,13]). In addition, there is a particular interest for the diagnostic and prognostic value of EVs as biomarkers [14,15].

2. The therapeutic potential of EVs

As the body of evidence for EVs being important mediators of intercellular signalling has been growing, so has their interest for

Abbreviations: EV, extracellular vesicle; MSC, mesenchymal stem cell; MVB, multivesicular body; PS, phosphatidylserine; GPI, glycosylphosphatidylinositol; PEG, polyethylene glycol; EGFR, epidermal growth factor receptor; RVG, rabies virus glycoprotein

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therapeutic use. The potential of using EVs in therapies can be summarised as follows: 1) Using endogenous EVs that possess innate therapeutic benefits, 2) EVs as therapeutic targets, 3) Modifying EVs for specific drug delivery systems.

2.1. Using endogenous EVs that possess innate therapeutic benefits

EVs harvested from numerous cell types have shown a remarkable capability to induce tissue regeneration and repair. Within the EV regenerative medicine field, there has been a particular focus on investigating EVs harvested from mesenchymal stem cells (MSCs). MSC-derived EVs have been implicated in the regeneration of, amongst others: bone and cartilage [16], cardiac tissue after myocardial infarction [17], renal tissue in models of acute and chronic kidney injury [18], and hepatic tissue after drug induced fibrosis [19]. Again, the functional effect of the EVs in most of these studies was attributed to the functional transfer of protective cargo to the injured cell. Similarly, EVs from embryonic stem cells can transfer early pluripotent transcription factors to hematopoietic progenitors to promote proliferation and survival [20]. Other non-stem cell derived EVs have demonstrated regenerative potential; for example, osteoblast derived EVs promoted osteogenic differentiation and biomineralisation in bone marrow MSCs [21], and stimulated antigen-presenting dendritic cells promoted EV-mediated oligodendrocyte myelination [22]. In addition, some studies have shown that EVs can indirectly elicit repair through EV-mediated immune regulation [23]. Similarly, EVs could modulate immune responses thereby giving a more favourable environment to promote healing or graft acceptance [24].

Some studies have even suggested that MSC-derived EVs may possess just as much regenerative promise as cell-based therapies [18]. Indeed, EVs may display innate potent regenerative potential without having the immunogenic, tumourigenic and shelf-life caveats that come with using MSC-based therapies [25]. There has been much interest in developing techniques for reproducible, sterile, large-scale EV purifications from MSCs for clinical applications [26,27]. There is also a growing interest in developing efficient ways of harvesting patient-specific EVs, where patient derived stem cells could be harvested, grown up and used to isolate patient specific regenerative EVs, which could be then used for matched regenerative purposes without risk of contamination or immune-rejection. There has been some success in clinical trials using autologous MSC-EVs to treat graft versus host disease [28], giving hope for the future therapeutic use of endogenous EVs in the clinic; however, there are no data available on the long-term patient consequences of EV treatments.

2.2. EVs as novel therapeutic targets

There are many reports of aberrant EV signalling in diseases. A greater understanding of the roles of EVs in pathogenesis could lead to novel EV therapeutics to exogenously block harmful, or restore protective, EV signalling. Within many cancer studies, EVs have produced pro-tumourigenic effects by transferring oncogenic nucleic acids and proteins (reviewed in [12]). EVs have been shown to contribute towards many pathogenic processes in cancer, including acquiring therapy resistance [29–31], stromal-tumour crosstalk [32], angiogenesis [4,33], establishing premetastatic niches [34,35] and modulating immune support [36]. In these cases, inhibiting the production, release or uptake of specific pro-tumourigenic EVs in the tumour microenvironment may be beneficial to halting metastasis and preventing invasive phenotypes. The silencing of Rab27a, a key Rab-GTPase involved in EV biogenesis, in various mouse tumour models was able to reduce tumour growth and metastasis, suggesting modulating EV signalling could be an effective therapeutic strategy [34,37]. In addition, GW4869, an inhibitor of neutral sphingomyelinase, is commonly used to block EV biogenesis in studies. However, both GW4869 and Rab27a silencing, are accompanied by off target effects such as altering homeostatic signalling and preventing lysosome/ lysosome-related organelle secretion [38], therefore they can interfere with normal cellular physiology. Alternatively, using pharmacological inhibitors of endocytosis to inhibit EV uptake may also be a therapeutic avenue to explore (as reviewed in [39–41]). However, any of these pharmacological inhibitors would be non-specific, both in terms of terms of their inability to distinguish between 'normal' and 'pathogenic' activity of EVs, as well as their off-target effects in non-EV related biological processes. A better understanding of the specific uptake mechanisms of EV subpopulations is therefore required to develop novel ways of targeting pathogenic EV communication.

2.3. EVs as drug delivery systems

EVs can deliver functional cargo to recipient cells, making them ideal candidates to be utilised as carriers for drug delivery. Similar to other synthetic nanocarriers, such as liposomes, polymersomes or lipid nanoparticles, they are able to encapsulate hydrophobic drugs and protect RNA/ protein cargo from enzymatic degradation to improve a drug's half-life in circulation by increasing the solubility, stability and persistence in the blood [42]. Endogenous EVs can reflect the lipid, protein and carbohydrate make up of parent cells; they therefore have low vehicle toxicity and low immunoreactivity. They boast several advantages over other synthetic nanocarriers, as they offer the unique intrinsic ability to cross biological barriers such as the blood brain barrier [43], have markers that reduce their immune clearance [42,44], and in some cases have innate targeting capability [35,45].

There has been some success in preclinical studies using EVs to deliver novel targeted therapies for cancers. MSC-EVs loaded with shRNA targeting an oncogenic form of KRAS [44] were able to reduce orthotopic human pancreas ductal adenocarcinoma growth in mice. Chemotherapeutic paclitaxel (PTX) containing EVs can be taken up by human pancreatic adenocarcinoma and prostate cancer cell lines to inhibit proliferation and induce cell death respectively [46,47]. In addition, doxorubicin loaded EVs are able to cross the blood brain barrier in a zebrafish cancer model to reduce the number of cancer cells [48]. In addition, there are a small number of ongoing clinical trials using EVs from dendritic cells (DCs) loaded with tumour-associated antigens for the treatment of malignancies (metastatic melanoma and non-small cell lung cancer [49,50]); these have passed safety trials and showed some therapeutic potential [51]. However, in order for endogenous or engineered EVs to be effective in the clinic, there is still much ground to uncover in the understanding of EV biology [52].

In addition to developing anti-cancer therapies, EVs are being explored in other therapeutic avenues. Recent developments include using EVs to deliver antibiotics [53] to treat localised infections, siRNA to target Huntington related genes [54], and antigens for vaccine development [55,56]. EVs have also successfully delivered CRISPR/Cas9 machinery [57] to recipient cells, to knock down specific genes paving the way for EVs to be used in the future for targeted gene editing.

Work is ongoing to improve the efficiency of using EVs as drug delivery systems. One way to do this is to increase the encapsulation efficiency of desired cargo into the EV. Like with other nanoparticles, pharmaceuticals can be passively or actively loaded into EVs. Passive techniques include simply incubating the desired cargo with the harvested EVs [47], or treating/transfecting a cell with a particular drug/ RNA and allowing the cell's own endosomal machinery to package cargo into EVs [46]. Alternatively, EVs can be actively loaded by electroporation, transfection, sonication, or extrusion (reviewed in [58,59]). Some novel active loading strategies have been developed, such as 'Targeted and Modular EV loading platform' (TAMEL), where the authors fused an EV-enriched protein (for example: CD63, Lamp2b, or Vesicular stomatitis virus glycoprotein; VSVG) to an RNA-binding domain (such as MS2 bacteriophage coat protein dimer), to load and deliver specific RNA to cells [60]. So far, EV loading strategies require further optimisation, they currently have variable encapsulation

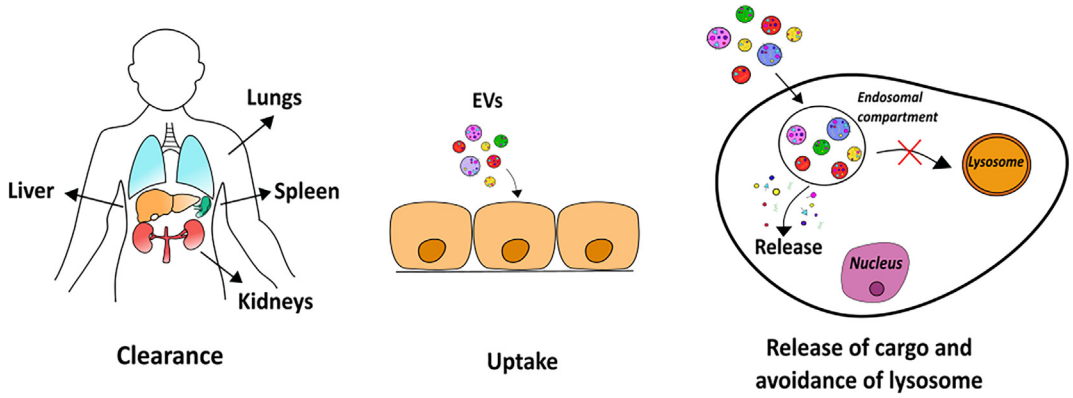

Fig. 1. Biological barriers to overcome to ensure therapeutic efficacy of EVs. Therapeutic EVs need to avoid rapid immune clearance and sequential bioaccumulation in non-targeted tissues, such as the liver, lungs, spleen and kidneys. EVs must persist in circulation, travel to their desired tissue destination, and cross the plasma membrane of their target cell by endocytosis or direct membrane fusion. Once endocytosed, EVs need to escape the endosomal compartment, and avoid lysosomal degradation before they can release their cargo. This cargo can then have the desired functional effect in the recipient cell, for example translation of protein.

efficiencies, and some active loading techniques interfere with the EV integrity, for example electroporation [61], which may disturb EV uptake or downstream functionality. A more thorough understanding of the molecular mechanisms of EV loading, uptake and utilisation of bioactive cargo is crucial to harness the full pharmaceutical potential of EVs.

3. Targeting and clearance of EVs

In order for EVs to be successfully applied as therapeutic vehicles there are several barriers and challenges which must be overcome (Fig. 1). These include: 1) Avoiding rapid clearance from the body, 2) Gaining access to the desired target tissue, 3) Being efficiently taken up by target cells, 4) Avoiding lysosomal degradation of the therapeutic cargo.

Understanding normal clearance and biodistribution of EVs is important for developing a more cohesive strategy for therapeutic delivery. Numerous studies have demonstrated that injection of EVs into the circulation of an animal leads to rapid clearance (within tens of minutes) from circulation [62,63]. Macrophage depletion can also result in an increased circulation time for EVs [64]. The primary sites of EV accumulation are the liver, spleen and lungs, where phagocytic cells remove EVs from circulation. This is also consistent with studies using injected nanoparticles [58,65]. Addition of 'don't eat me' signals such as CD47 [44] or PEGylation of EVs [66] can increase time in circulation. Most exogenous biodistribution studies rely on a bolus injection of large quantities of EVs, which might not represent typical *in vivo* localisation of EVs. Interestingly, the sites of EV accumulation may vary according to the location of administration [67]. For example, whereas tail vein injections lead to EV clearance in the spleen and liver, subcutaneously administered EVs are more likely to amass in the lymphatic system [62,67–69]. In addition, there is evidence EVs being excreted from the urinary and digestive system [70,71], suggesting that therapeutic EVs may also be cleared by these mechanisms. To increase accumulation of therapeutic EVs at the target site further, research is needed to understand how different EVs (endogenous vs modified; and different subpopulations) are cleared from circulation in these different ways.

Therapeutic EVs must successfully reach their target tissue to have their intended effect. Depending on the site of administration this may involve traversing the systemic circulation to capillaries where they can extravasate [72] or transcytose through the vascular endothelium, however how EVs achieve this is not well characterised. EVs have been shown to roll, and attach to vascular endothelium, before being endocytosed, in live EV tracking zebrafish studies [3,73], however how these EVs manage to escape the endothelium to reach deeper tissues is largely unknown. In addition, several studies have shown EVs successfully delivering cargo to the neuronal tissue across the blood brain barrier [43,48,72]. Understanding how EVs can cross other biological barriers such as epithelium, deep muscle and skin, is important to develop targeted EV therapies without local administration.

Increased delivery to diseased tissue has been observed in some studies. For example, increased levels of inflammation has been shown to increase the ability of EVs to cross the blood brain barrier [72] and be taken up by neuronal cells [74,75]. Myocardial infarction also leads to increased inflammation and vascular leakiness which leads to increased access for EVs [76]. Some studies have also shown an increase in EV uptake in xenografted tumours compared to surrounding healthy tissue, mostly likely caused by increased vascular fenestration around the tumour [67]. Nevertheless, the numbers of EVs that accumulate in such sites is still much lower than those being cleared in the liver and spleen. Therefore, specific targeting strategies are being developed to increase the probability that EVs reach the target tissue.

To increase the likelihood of EVs accumulating at a desired location, EVs can be modified in a variety of ways, which lead to stronger affinities for target cells [77]. Using an engineered protein containing a phosphatidylserine (PS) binding module and a nanobody with specificity for epidermal growth factor receptor (EGFR), Koojimans and colleagues (2018) were able to decorate the surface of EVs (via the specific binding of the PS-binding module) and increase the ability of the EVs to be specifically taken up by EGFR-overexpressing cells [78]. Similarly, engineered EVs containing PS-binding and HER2-targeting proteins have been shown to increase delivery to HER2-expressing cells [79]. Alternative cloaking strategies have involved the use of streptavidin and biotin to anchor specific target modalities to EVs [80] or glycosylphosphatidylinositol (GPI)-anchored moieties [81]. Using an alternative targeting method of fusing Lamp2b (an EV protein) to a neuronal-specific rabies virus glycoprotein (RVG), it was shown that systemically delivered RVG EVs loaded with a BACE-1 siRNA were successfully delivered to mice cortices [43]. Such studies are beginning to show that it may be feasible to specifically target EVs to desired tissues. Several studies have revealed EVs have endogenous proteins on their surfaces which result in natural specific targeting. Integrins on EVs have been shown to predict where which site malignant cells may metastasise to [35]. The presence of specific integrins on the EVs allowed targeting to specific cells in the lungs, liver or brain. In addition, members of the tetraspanin family have been implicated in integrin targeting to specific tissue, Tspan8 coupled with α4 integrin was able to direct EVs to the CD54 positive pancreatic cells [82]. Better understanding of these 'rules of endogenous targeting' should also lead to improved specificity of engineered EVs.

4. The barriers of uptake and delivery

The next barrier that EVs must traverse is the plasma membrane. There are numerous ways in which EVs are internalised by cells, including clathrin-mediated endocytosis, caveolin-mediated endocytosis, macropinocytosis, phagocytosis and direct fusion [39,40]. EV uptake studies have collectively shown that internalisation is an active process, which uses a combination of classical endocytic pathways. Many studies have utilised a combination of RNAi, antibodies and pharmacological inhibitors to inhibit the classical endocytic pathways and pinpoint the exact contribution of each type of endocytosis. However, the current evidence points to not one individual but a combination of uptake mechanisms that facilitate EVs uptake. Discrepancies between studies may be due to differences in the cell types being used and the precise methodological approach. Therefore, much work is needed to fully understand the mechanisms of their internalisation.

Another factor to consider in EV uptake is the role of heterogeneity. EV preparations are inherently heterogeneous [83–85], and what is not currently known is whether all EV subtypes are able to access cells through the same uptake mechanisms, or whether the different entry mechanisms restrict access to specific subtypes. Loading therapeutic molecules into one EV subtype may therefore restrict entry of the cargo to specific endocytic pathways.

Whilst much is known about the mechanisms of EV uptake, how EV cargo escapes the endosomal compartment is less well characterised. Several studies have demonstrated that EV cargo can be functionally delivered, leading to, for example, translation of mRNAs and repression of miRNA targets [4,5]. However, the EV cargo transfer efficiency appears to be low [60,86]. Despite having some knowledge of the cell machinery required in the donor cell, for endogenous EV loading (reviewed in [40]), there is little known about the exact molecular machinery required in the recipient cell to unload selected EV cargo. One study has shown that EVs are able to track along the cytoskeleton and towards the endoplasmic reticulum [87]; the mechanisms and functional consequences of this are not clear. Most studies have shown that following endocytic uptake, EVs are delivered to the early endosome, which then matures into the late endosome [60,86,88]. The late endosome can further mature into a multivesicular body and can fuse with the lysosome, leading to the lysosomal degradation of its contents. Some *in vivo* studies have shown the accumulation of EVs in endo-lysosomal compartments [3,73]. It is not clear how the EV can avoid this lysosome-mediated destruction of its cargo. One possibility is that the EV can fuse with the endosomal membrane prior to lysosomal delivery. However, how this is regulated remains to be delineated. Bacteria and viruses have known mechanisms to escape the endosome (reviewed in [89]). They undergo membrane pore formation and fusion events at lower late-endosomal pHs, which are mediated by fusogenic proteins or agents such as haemagglutinin, gp41, TAT, polyethylenimine (PEI), and cholera toxin. New avenues for engineered EVs could explore the use of viral/ bacterial proteins that are capable of enforcing endosomal escape.

5. Future outlook for EV-mediated drug delivery

As has been described above, there are numerous technical challenges that need to be overcome for the potential of EV-mediated drug delivery to be realised. These include better loading of therapeutics into vesicles, avoidance of clearance from circulation, targeting to specific cells and efficient uptake and delivery of the therapeutic cargo. These are technical challenges that require more basic and applied research.

There are also a number of challenges associated with working on EVs in general [90]. There is an urgent need for standardisation of the isolation, quantification and characterisation of EVs [91]. The method of isolation can influence the purity of EV preparations, and could potentially affect their suitability for efficiency of therapeutic loading and/or their efficacy in the *in vivo* setting. The choice of EV type as a carrier is also important, particularly when considering the need to scale up production. Indeed, one of the major challenges for translating EVs successfully into the clinic is to ensure efficient scale up of clinically effective EVs. Currently the EV field lacks reproducible ways of generating large batches of EVs isolated from a single source. Technological advances of sterile isolation methods, which are low on impurities of bacterial/viral/ host contaminants should be a priority. Some studies have been attempting to address this issue [26].

Recent advances in our understanding of EVs and EV-like nanoparticles reveal that the EV field is more complex than previously thought. Larger oncosomes, EVs between 1-10 μm in size that possess pro-tumourigenic activity, have challenged the field's previously held view on the size limits of EVs [92]. Conversely, exomeres, sub-50 nm non-membrane bound nanoparticles, have also been recently highlighted as an additional class of EVs [93]. However, it is too early to know if exomeres or oncosomes hold therapeutic benefit in their own right. In addition, recent studies have challenged previously held views about EV cargo and biogenesis [85,94], including the absence of DNA in EVs. Clearly, further understanding of EV biology would help us to harness their true therapeutic potential.

Another important challenge is to ensure that EV-based therapeutics meet the rigorous requirements for clinical approval by regulatory bodies. As EV-based therapeutics are less mature than other types of nanomedicine, the regulatory considerations have not been tackled as thoroughly to date [95]. However, consideration of the needs for clinical assays and therapeutic products in the EV field is emerging [26,96]. Methods of EV production, batch-to-batch uniformity and the use of activity assays for standardising production are all important considerations for the therapeutic use of EVs [52,97–99]. This area needs to be further developed to ensure the regulatory needs of EV-based therapeutics are met.

EVs are the cells' own mechanism for transferring various types of cargo all in one package. It makes sense to harness their potential in the therapeutic setting. Whilst numerous technical challenges exist to ensure efficient delivery of therapeutics, the progress that has been made in the last decade makes this a feasible and exciting approach for many diseases.

6. Declarations of interest

None.

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