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EDITORIAL

Hitting the Bullseye: Are extracellular vesicles on target?

1 | INTRODUCTION

Great strides have been made in advancing extracellular vesicles (EVs) to clinical testing (Hu, Wolfram, & Srivastava, 2020; Lener et al., 2015, Reiner et al., 2017; Rohde, Pachler, & Gimona, 2019). By late 2020, approximately 250 trials that utilize EVs in some way had been registered in clinicaltrials.gov. Diagnostic, prognostic, and monitoring uses of EVs are evident in these registrations as well as applications of EVs in therapeutics. Interest in EVs stems in part from their biology (Yáñez-Mó et al., 2015). They are involved in natural processes of communication in the body and have a perceived safety profile that features low immunogenicity. Additionally, EVs are 'targetable'. Display of specific proteins, and possibly other biomolecules, allows EVs to be sorted to certain cell types and tissues or away from undesired recipients (Ferrantelli, Chiozzini, Leone, Manfredi, & Federico, 2020; Pirisinu et al., 2020; Walker et al., 2019). EV engineering, by manipulating the EV source or by altering EVs post-production, can be used to enhance such targeting. Modified EVs have been used for some time as delivery vehicles for small molecule drugs and natural products, short hairpin RNA (shRNA), short interfering (siRNA), plasmid DNA and microRNAs (Sun et al., 2010; Tian et al., 2014) (Figure 1). However, a key factor in the success of this and other EV therapies is whether and how EVs can be targeted to, or away from, specific cells.

In this editorial, we offer our perspectives on EV targeting as organizers and participants in an ISEV Workshop on the topic, which has been followed by extensive subsequent discussions. We first review several features of EVs that make them suitable and desirable as therapeutic platforms. We then provide several examples of strategies to target EVs to specific cell types and to avoid recognition by other cells. Along the way, the concept and implications of 'targeting' are examined critically. In some applications, success of an EV therapy, like real estate, may be all about 'location, location, location': physically applying the EVs in the right place. And of course, many aspects of EV production influence eventual use and efficacy. Finally, we examine how targeting (or selective retention) of EVs can be confirmed *in vivo*.

2 | WHY ARE EVS ADVANTAGEOUS AS THERAPEUTICS?

EVs are nano-sized membranous structures released by cells into the extracellular space, including most, if not all, body fluids (Minciacchi, Freeman, & Di Vizio, 2015; Witwer & Théry, 2019). Their multiple biological functions range from removal of harmful materials from the cell to trophic support and to mediation of intercellular and interorgan communication. EVs can serve as messengers by binding to signalling receptors on recipient cells or via transfer of functional cargo such as microRNAs (miRNAs), mRNAs, proteins and lipids. EVs can infiltrate biological barriers through transcytotic processes or, for the smaller EVs, through small vessel fenestrations, e.g. in the tumour microenvironment. Furthermore, EVs generally show minimal native immunogenicity when compared with artificial nanoparticles, and the immune response can be avoided using autologous EVs (Villa, Quarto, & Tasso, 2019). EVs can be modified by loading specific cargo or by changing EV surface molecules to target the EV to, or away from, specific cell types (Patel, Santoro, Born, Fisher, & Jay, 2018) (Figure 1).

The use of EVs is currently being evaluated as a therapeutic strategy for treatment of variety of diseases including cancers (Pirisinu et al., 2020; Walker et al., 2019), cardiovascular diseases (De Abreu et al., 2020), neurogenerative diseases (Ferrantelli et al., 2020) and diabetes (Noren Hooten & Evans, 2020; Xiao et al., 2019). Many of these pre-clinical studies have shown promise in *in vivo* models and use EVs to deliver specific cargo such as miRNAs, siRNAs and proteins. Most recently, several interventional clinical trials have begun to assess the efficacy of EVs as treatment for COVID-19, acute myocardial infarction, acute ischemic stroke, periodontitis and several other conditions (clinicaltrials.gov). However, it should be noted that many of these trials are limited in numbers of participants, scope and detailed data availability. Many commercial companies have also pursued EVs as therapeutics (Popowski et al., 2020).

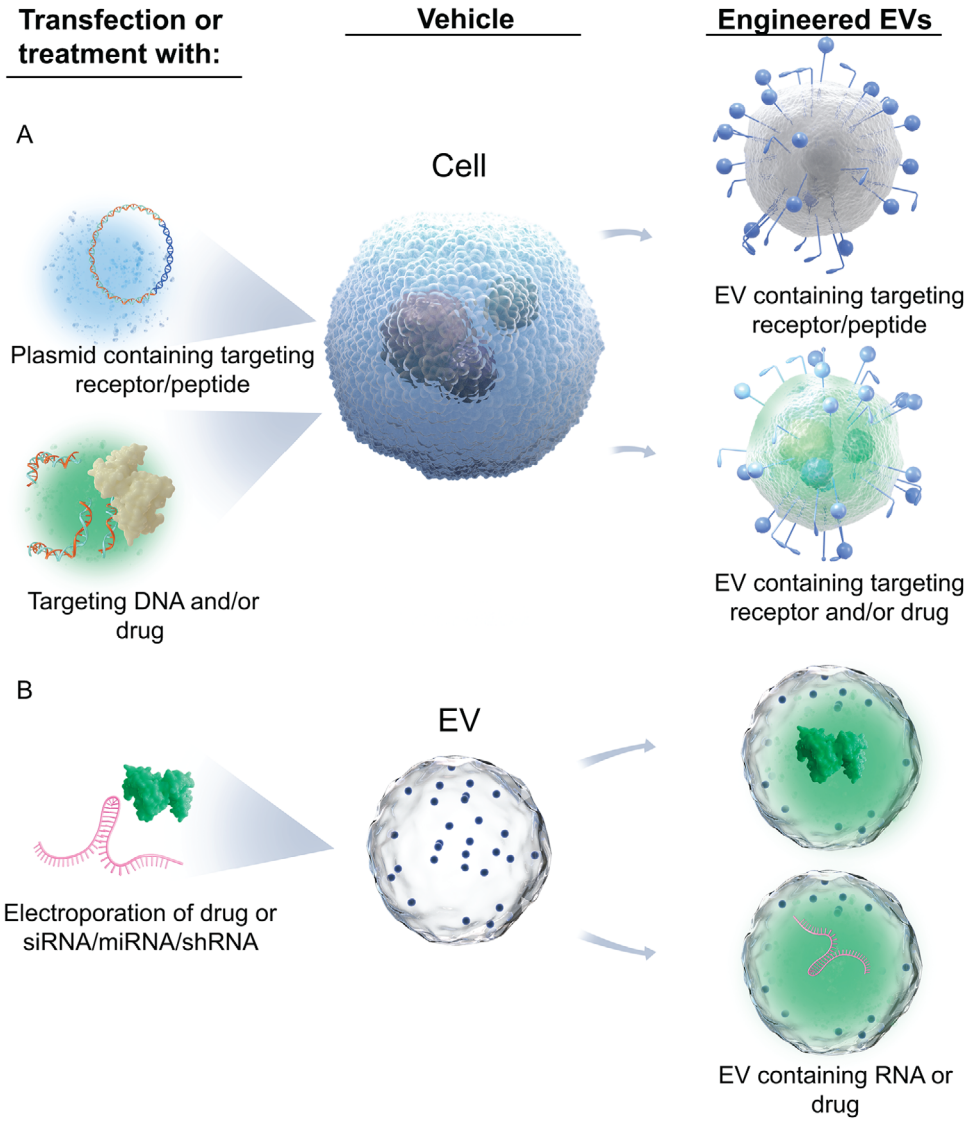

FIGURE 1 Current strategies for engineering EVs. (a) Cells can be transfected with plasmids containing targeting receptors and/or peptides. EVs bearing the targeting receptor and/or peptide can then be isolated from these cells. Cells can also be treated with a drug that then is encapsulated into an EV for delivery. In addition, cells can be both transfected with targeting plasmid and treated with a therapeutic drug for EV delivery. (b) EVs can be electroporated (or equivalent technique) with a drug of interest or RNA molecule (siRNA, miRNA or shRNA) for incorporation into the EV. This figure was created with help from Nicolas Fernandez.

Although most therapeutic approaches aim to avoid immune responses, some applications seek to stimulate specific immune signalling in a 'targeted' manner. This is illustrated by a clinical trial of EVs from dendritic cells as a potential cancer vaccine (Besse et al., 2016). Antigens can be engineered onto the EV surfaces to target antigen presenting cells in a strategy that may be synergistic with existing immune checkpoint therapies. Genetically engineered T cells expressing a chimeric antigen receptor (CAR) have been shown to release EVs that carry functional CAR on their surface (Fu et al., 2019). In addition, vaccination strategies using EVs are emerging in the fight against infectious diseases (Marcilla et al., 2014; Olivier & Fernandez-Prada, 2019). All of these features strongly support the use of EVs as therapeutic entities.

3 | TARGETING AND SELECTIVE RETENTION

Targeting EVs to specific cell types could indeed be considered a holy grail of EV therapeutics, since cell specificity reduces the necessary dose and minimizes off-target effects. However, we should be clear that the word 'targeting' is used colloquially. The typical EV cannot move towards a destination as a result of interpreting signals, for example, by crawling along a chemical

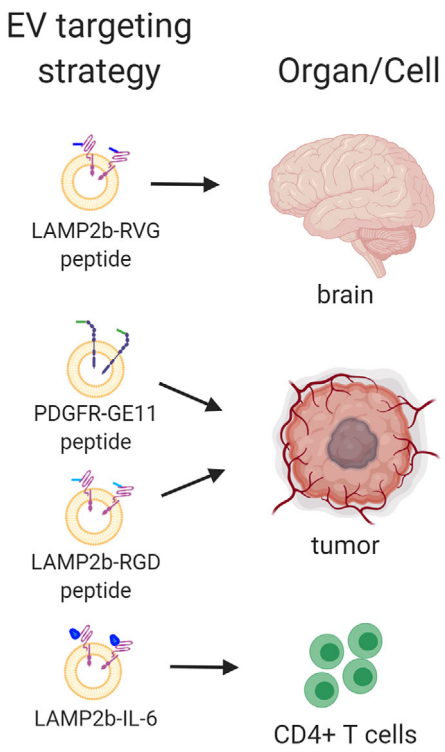

FIGURE 2 Strategies for EV targeting to specific cells or organs. Examples of targeting strategies for delivery of EVs to target organs/cells. PDGFR: platelet derived growth factor receptor; LAMP2: Lysosomal Associated Membrane Protein 2. This figure was created with BioRender.com.

gradient, so the EV cannot truly 'home' to a specific cell. Instead, the word 'targeting' refers more accurately to 'selective retention' or 'capture' by the target cell.

Selective retention is typically achieved by receptor-ligand pairing. A molecule, usually a receptor, expressed on a specific cell type or during specific conditions is identified. Next, a ligand is displayed on the EV surface, usually a peptide or protein. Transmembrane Lamp2b has been a workhorse for this strategy (Figure 2). For example, a neuron-specific RVG peptide-Lamp2b fusion construct retains EVs in the brain (Alvarez-Erviti et al., 2011). Modified EVs expressing the peptide GE11, which binds specifically to the EGFR, fused to the PDGFR transmembrane domain, target EGFR-expressing breast cancer cells (Ohno et al., 2013). A RGD peptide fused to Lamp2b interacts with αv integrin on the surface of breast tumours, permitting EV delivery of doxorubicin or therapeutic siRNA (Figure 2) (Tian et al., 2014). An IL-16 domain fused to Lamp2b targets EVs to $CD4^+$ T cells (Tang et al., 2018). Lamp2b is of course not the only EV protein that can be used, and other strategies for targeting have also been employed (Pirisinu et al., 2020; Walker et al., 2019). Other approaches to modify EVs and increase their affinity for selective organs could also be considered (reviewed in (Murphy et al., 2019)), including the use of aptamers (Hosseini Shamili et al., 2019; Wang et al., 2017). In addition, heparin sulfate proteoglycans on cancer cells have been shown to be important for EV targeting and uptake (Christianson, Svensson, Van Kuppevelt, Li, & Belting, 2013).

Alternatively, selective retention may be achieved in the opposite direction, with receptor-ligand pairing, whereby the receptor is found on the EV surface and the ligand in the targeted cell. Specific receptors such as integrins have been shown to mediate EV targeting and have been reported to direct organ targeting in models of metastatic disease (Hoshino et al., 2015). Overall, cell- or organ-specific successful targeting *in vivo* will require adapting and tailoring future investigations to specific needs.

4 | ON THE FLIP SIDE: AVOIDING INTERACTIONS AND INCREASING STABILITY

It is estimated that the half-life of exogenous EVs introduced into the circulation is relatively short (~10 min) after intravenous injection into mice (Kooijmans et al., 2016). Therefore, in addition to targeting EVs 'to' a cell, it might be desirable to interfere with their clearance. Here polyethylene glycol (PEG)-conjugated EVs have shown improved stability (Kooijmans et al., 2016). Blocking scavenger receptors reduces EV accumulation in liver, giving EVs a better chance to reach target organs (Watson et al., 2016). Similarly, the presence of CD47 as a 'don't eat me' signal to macrophages on the surface of EVs also increases EV half-life (Kamerkar et al., 2017); the CD47-SIRPα pathway is also currently being explored, using engineered EVs harbouring SIRPα, to augment tumour cell phagocytosis and effective priming of an anti-tumour T cell response (Koh et al., 2017). However,

alternative pathways are used by aggressive cancers to avoid clearance by modifying monocytes. These cancers produce EVs which express increased levels of integrins on their surface and target monocytes to induce their differentiation into pro-tumorigenic macrophages (Lu et al., 2018). Appropriately engineered EVs thus may be more stable in the body compared with liposomes (Kamerkar et al., 2017). We expect that additional important information for human patients will be derived from the pharmacokinetic profiles of EVs in the different phase I trials.

5 | PUT THEM IN THE RIGHT PLACE!

To the extent possible, administering EVs at the site of intended action will enhance selective retention and help to avoid clearance. Many studies use intravenous delivery of EVs, but this results in delivery predominantly to just a few organs, especially lung and liver as well as bone marrow, spleen and kidney. Introducing EVs by different routes, or even directly to the target site by application (e.g. skin wound healing, eye) or tissue injection avoids rapid clearance and maximizes dosage. This aspect also relates to the therapeutic dosage since thus far, there is not yet a definitive appropriate concentration of EVs to be used for *in vivo* experiments both in humans and in animal models.

The therapeutic efficacy of EVs targeting KRAS has been optimized in one experimental setting (Kamerkar et al., 2017; Mendt et al., 2018), in which engineered EVs purified from normal human foreskin fibroblasts and loaded by electroporation with siRNA against mutant KRAS (KrasG12D) are being used as a therapeutic for metastatic pancreatic cancer in a clinical trial. This approach has slowed progression and improved survival in mouse models (Kamerkar et al., 2017), and the trial will test dosage and pharmacokinetics, and assess disease outcomes (NCT03608631) (Figure 1). However, this approach does not intrinsically target the EV to tumour cells, but instead has a therapeutic payload that would presumably affect only those recipient cells that harbour a mutant KRAS.

In general, the therapeutic efficacy of any specific EV needs to be empirically optimized in each experimental setting via serial titrations of EVs. For therapeutic effects *in vivo*, though, the number of EVs required may actually be significantly lower than what would be predicted from *in vitro* experiments. Over-dosage may be ineffective or even have deleterious effects, since some EVs carry coagulative factors (Yáñez-Mó et al., 2015) that might induce local or disseminated thrombosis.

6 | PRODUCTION OF EVS FOR TARGETING

Beyond obvious process considerations such as production scaling and satisfying regulatory requirements for clinical use, the ability of an EV preparation to achieve targeting will depend on the separation method that is used. We would like to emphasize two important points. First is the concept of 'pure enough' versus 'too pure'. EVs from any biological source are heterogeneous, and it is likely impossible to obtain a 100% pure population of EVs or of a particular EV subtype such as exosomes. Comprehensive characterization is encouraged (Théry et al., 2018) to establish the presence of EVs and the absence of expected contaminant classes, and these studies will inform proper nomenclature and reporting. However, some 'contaminants' that are directly or indirectly associated and co-purified with EVs could play important functional roles in modulating targeting. The important outcome is achieving consistent targeting and therapeutic effect, even if the EV preparation is not 100% pure. Second in addition to purity considerations, some separation methods may be better for retaining targeting functions than others by virtue of retaining membrane topology and vesicular surface decoration as well as avoiding introduction of exogenous materials (e.g. polymer precipitants and gradient materials (Jeurissen et al., 2017; Szatanek, Baran, Siedlar, & Baj-Krzyworzeka, 2015)) that might interfere with function. As alternative methods to ultracentrifugation, which is limited in processing volume and can induce EV aggregation or even membrane-flipping damage, size exclusion chromatography (SEC) (De Menezes-Neto et al., 2015) and tangential flow filtration (TFF) are gentler and scalable approaches (Busatto et al., 2018). As always, methods should follow consensus standards and reporting (EV-TRACK Consortium, Van Deun J, et al; Théry et al., 2018).

Fully or partially synthetic EVs and EV-mimicking nanoparticles from natural sources have been proposed to overcome issues of heterogeneity and scalability that affect native EVs. If all active components of a therapeutic EV type are identified, they might be prepared in the laboratory and combined into a synthetic EV product that is highly pure and reproducible (Lozano-Andrés et al., 2019; Surman, Drożdż, Stępień, & Przybyło, 2019). However, because of the complex nature of EVs, making a truly 'recombinant' synthetic EV (as opposed to a defined virus-like EV particle (Geeurickx et al., 2019)) may be a difficult task. It is also possible to prepare EVs and add synthetic components in a plug-and-play strategy (Antes et al., 2018; Kooijmans, Gitz-Francois, Schiffelers, & Vader, 2018; Van Den Berg Van Saparoea, Houben, Kuijl, Luirink, & Jong, 2020), combining the advantages of native EVs with the versatility of recombinant systems. Another proposed approach is to use materials harvested from highly abundant natural sources such as plants and cow milk (Pocsfalvi et al., 2018; Raimondo, Giavaresi, Lorico, & Alessandro, 2019; Somiya, Yoshioka, & Ochiya, 2018). In some cases, native EVs can be separated from the source material (as for milk), but in others (especially for plants), tissues are destructively processed to produce tissue-derived EV-mimetic nanoparticles. The product is thus similar to the results of extrusion of mammalian cells or bacteria (Kim et al., 2017; Lunavat et al., 2016). EV mimetics from

natural sources will be different from native EVs to the extent that specific biogenesis pathways impress characteristics on EVs. They will include regions of membrane with 'flipped' topology as well as components from cellular compartments (nucleus, mitochondria, etc.) that are typically uncommon in EVs compared with the cell (Russell et al., 2019). Incorporation of danger-associated molecular patterns (DAMPS) may complicate the safety profile of such EVs. However, these particles could also be engineered pre- or post-production to optimize safety profiles and targeting.

7 | HOW DO WE KNOW THE EV HAS REACHED ITS TARGET?

Many studies of EV targeting have relied on lipid dyes, showing that EVs accumulate in injured tissues, such as spleen and bone marrow after radiation or in kidney after acute injury (Grange et al., 2014; Wen et al., 2016). However, better labelling approaches are needed, since lipid dyes are known to self-aggregate as EV-like micelles, they can bind to protein aggregates, and may also be promiscuous, greatly complicating interpretations (Simonsen, 2019). Intraluminal dyes, such as CFSE, also are used but have not been yet utilized extensively for *in vivo* studies. An alternative approach is to engineer EVs with fluorescent or luminescent tetraspanin fusion proteins (Hyenne et al., 2019; Verweij et al., 2019). EV incorporation of these tags must of course be efficient and the tags themselves bright enough, for detection. Considering that the surface of a 100 nm diameter EV would be 1/250,000 of the surface of a 10 micron diameter cell, the limited surface of an EV would be able to accommodate at a maximum only a few dozen of the most abundant surface marker such as tetraspanins, in comparison with millions of copies in a single cell. It must also be feasible to provide a non-toxic substrate in the case of luminescent reporters. Although a majority of reports so far analysing EV biodistribution and targeting *in vivo* rely on fluorescent methods (for a thorough review see (Yi et al., 2020)), other labelling strategies include radiolabelling (Chuo, Chien, & Lai, 2018) and gold nanoparticle-based double-labelling (Lara et al., 2020). Functional delivery of EV cargo *in vivo* has also been demonstrated by applying Cre protein-carrying EVs to target cells carrying a floxed fluorescent reporter (Zomer, Steenbeek, Maynard, & Van Rheenen, 2016). This system represents a rigorous *in vivo* readout model, although recombination efficiency is somewhat low. In a reverse manner, a floxed-fluorescent CD63 reporter mouse has been generated to allow cell-specific labelling of circulating EVs by crossing with lineage-expressing Cre models (Mccann et al., 2020). Despite these important advances, detection of *in vivo* EV uptake would benefit from further optimization and developments to enhance sensitivity.

8 | CONCLUSIONS

The strategies described above will pave the way for new clinical trials, which will require new investigations to determine EV pharmacokinetics (absorption, distribution, metabolism and excretion, ADME), dosage, routes of administration, formulation and stability for clinical application. Additional investigations will be needed to identify specific physiologically based pharmacokinetic models to evaluate EV effects. The results of these trials will be critical for determining the efficacy of EV-based therapies. EVs cannot truly 'home' to a specific cell, and researchers will likely need to focus on selective retention of EVs by the target cell while also considering plausible strategies for blocking non-specific uptake of EVs by off-target cells. Both of these aims may be achieved by engineering EVs specifically for targeting studies, but these aims require a great deal of optimization for their creation, their separation from fluids, and appropriate techniques for ensuring that EVs have indeed reached their target cells.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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