

Experimental limitations of extracellular vesicle-based therapies for the treatment of myocardial infarction ^{☆,☆☆,★}

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ABSTRACT

Extracellular vesicles (EVs) are particles secreted by a vast variety of cells and are often recognised to mimic the properties of their parent cell, as such those derived from developmental sources hold promise for the treatment of various diseases including myocardial infarction (MI). Here we review the experimental approaches taken for assessing the therapeutic efficacy of EVs for MI and find overt shortcomings regarding purity of isolated EVs, quantitation, dosing, EV labelling/uptake, route of administration and use of appropriate controls that renders much of the data uninterpretable. Overall, the EV/MI field has suffered from experimental approaches that are not fully standardised or validated. Fundamental improvements in EV study design are required to improve interpretation of efficacy and to ensure reproducibility and comparability across preclinical MI studies.

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Introduction

Myocardial infarction (MI) presents a major public health problem with high rates of morbidity and mortality worldwide [1]. Coronary artery occlusion leads to extensive and irreversible loss of cardiomyocytes and subsequent replacement of functional myocardium with non-contractile fibrotic tissue. Although current therapies, targeted at restoring blood flow and assisting the survived heart muscle, reduce mortality they do not address the underlying loss of functional tissue [2]. Consequently, pressure overload on the remaining muscle ensues leading to pathological remodelling and heart failure [3]. The global incidence of heart disease, compounded by limitations of current treatments, highlights the inability of the human heart to functionally repair itself as a significant area of unmet clinical need.

Strategies aimed at promoting cardiac regeneration have been an area of extensive research focus with cell-based approaches having progressed to clinical trials. However, poor cell-survival, migration and integration into host tissue have to-date prevented the approval of cell therapy in patients for MI [4,5]. The interest in paracrine factors for MI treatment increased after it was demonstrated that the pre-clinical benefits of mesenchymal stem cell (MSC) therapy post-MI could be recapitulated by administering the cells' conditioned medium alone [6]. Extracellular vesicles (EVs) were identified to be the major component of the stem cell secretome responsible for the observed increase in cardiac function [7]. EVs are phospholipid-bound particles that contain diverse combinations of proteins (including enzymes, growth factors, receptors and cytokines) as well as lipids, coding and non-coding RNAs and metabolites [8–10]. EVs are secreted by parent cells and have the potential to influence the phenotype of recipient cells. As EVs are often recognised to mimic the properties of their parent cell, EVs derived from developmental sources in which embryonic potential might be mimicked have recently become an area of intense focus for the treatment of MI. EV-based therapies may present a scalable treatment option that negates issues associated with cell therapy such as survival, integration, migration and immunogenicity.

The therapeutic use of EVs has shown great promise and as such the field has expanded rapidly but standardisation of exper-

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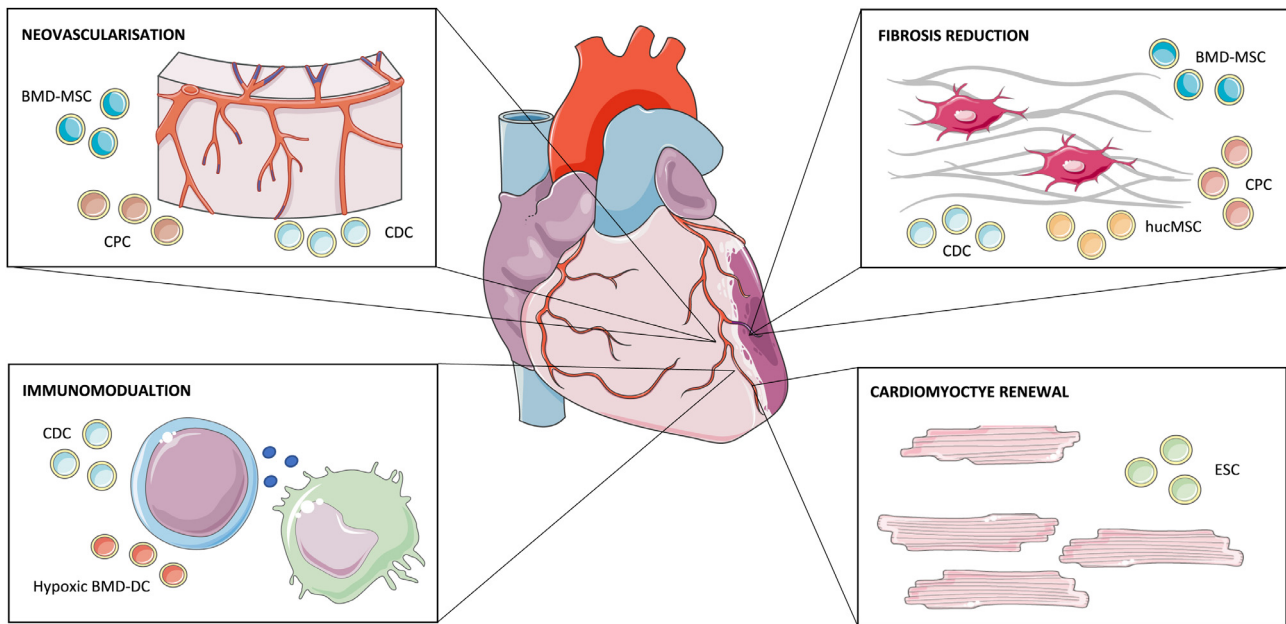


Fig. 1. Relevance of Extracellular vesicle (EV)-based therapies for myocardial infarction (MI). EV treatments have been implicated in targeting the major areas of therapeutic interest in MI, including; neovascularisation, fibrosis reduction, immunomodulation and cardiomyocyte renewal. EVs used in these studies were derived from; bone marrow-derived mesenchymal stem cells; BMD-MSC, cardiac progenitor cells; CPC, human umbilical cord-derived mesenchymal stem cells, hucMSC; cardiosphere-derived cells, CDC; bone marrow-derived dendritic cell, BMD-DC and embryonic stem cells; ESC.

imental application has struggled to keep up; technical variation and limitations in EV isolation, their basic characterisation and accurate dosing regimens often confound interpretation of findings. Efforts have been made to remedy the lack of cohesion within the field, including proposals of common nomenclature and baseline requirements for experimental reporting [11–13]. However, even studies conducted in accordance to these guidelines and within the same disease context are often incomparable. This is particularly evident when applied to cardiovascular disease models and most notably in the context of acute MI (AMI). Although EV treatments have been implicated in key areas of therapeutic interest in MI (Fig. 1), technical limitations and shortcomings have made data difficult to interpret and the field as a whole could benefit from a more defined preclinical framework. Table 1 highlights studies investigating the potential of EVs for treating MI in preclinical animal models. The efficacy and biology of EV therapies for cardiovascular disease have previously been reviewed elsewhere [14–17], here our focus is on the technical differences between studies and the implication these have on the interpretation of the data and the progress of EVs as clinical solutions to AMI.

Classifications of EVs

The term EV pertains to all vesicles secreted by cells but EVs are typically categorised into three main groups; exosomes, microvesicles and apoptotic bodies. After prolonged ambiguity regarding EV classification, standardised nomenclature was proposed by Thery *et al* in 2009 [13] and further refined by Gyorgy *et al* in 2011 [12]. These groups suggested EVs be defined as follows; exosomes are 50 - 100 nm in diameter, generated by exocytosis of multivesicular bodies; microvesicles are 100 - 1000 nm in diameter formed by regulated release by budding/blebbing of the plasma membrane; and apoptotic bodies are EVs 1-5 μ m in diameter released as blebs from cells undergoing apoptosis. However, these definitions are approximate with budding and exocytosed EVs of the same size range having been observed [12,13]. Furthermore, the functional differences between exosomes and microvesicles are poorly understood. As exosomes are typically pursued for therapeutic applica-

tion, methods for isolation have centered around selecting biophysical properties, e.g. size and density characteristics, thought to be specific to exosomes populations.

Methods of EV isolation

Numerous methodologies exist for the isolation of EVs that can be grouped into five main categories based on properties selected: density/sedimentation (ultracentrifugation; UC), size exclusion (size exclusion chromatography; SEC; ultrafiltration; UF), volume exclusion (precipitation), immunoaffinity and microfluidics-based isolation. These techniques have been reviewed for general application recently by Li *et al* [18], with respect to recovery of EVs for application during MI; UC, SEC and precipitation-based techniques have been most extensively applied in preclinical studies. Below we discuss the implications of the isolation methods used in the studies outlined in Table 1.

Contamination of non-EV material is by far the greatest variable associated with isolation method with significant potential for non-specific downstream experimental effects. Comparative studies of EV isolation techniques in human plasma and squamous Non-Small-Cell Lung Cancer cells (NSCLC) found elevated EV particle numbers recovered with precipitation-based techniques compared to SEC-isolation [19,20]. However, protein amount was found to be disproportionately elevated in precipitated NSCLC EVs with a four-eight-fold-increase in particle number to protein content ratio observed compared to SEC-isolated EVs [20] and whole protein content was drastically increased in EVs recovered from serum using polyethylene glycol (PEG)-precipitation (21.1 mg) compared to SEC isolation (0.3 mg) [19]. A similar pattern is observed for PEG-based compared to commercial precipitation techniques in Human embryonic kidney 293T cells (HEK293T)-derived EVs [21]. These observations are pertinent to EV-based therapies for MI as EVs from numerous sources; cardiac progenitor cells (CPC) [22], bone marrow-derived (BM) dendritic cells [23], induced pluripotent stem (iPS) cells [24], cardiosphere-derived cells (CDC) [25], mesenchymal stem cells (MSC) [26], BM endothelial progenitor cells [27] and serum [28] have utilised precipitation-based iso-

Table 1
Comparison of Extracellular Vesicle (EV) applications in preclinical studies of myocardial infarction (MI).

EV Source	Isolation method	Uptake assessment	<i>In vitro</i> dose	MI procedure	ROA, <i>in vivo</i> dose and controls	<i>In vivo</i> Outcomes
Mouse-derived cardiac progenitor cells (CPC) [22]	PEG-precipitation	PKH26 dye, <i>in vitro</i> , 12 hr post-delivery	Not reported	Male, C57BL/6 mice 25–30g mice. 45 min I/R via LAD artery ligation	i.m. injection at time of MI. EV from 5×10^5 cells in 25 μ l per mouse. Controls: PBS	EV treatment reduced apoptotic staining in cardiac cross sections of treated mice taken 24 hrs post-MI.
Mouse bone marrow-derived dendritic cells (hypoxia activated vs normoxic) [23]	Commercial precipitation kit ExoQuick	PKH67 and DiR dye, <i>in vivo</i> , between 1 h and 7 days post-delivery.	100 μ l EV in 500 μ l medium	Male, C57BL/6, 8-week-old mice. Permanent LAD artery ligation	t.v. injections 1-day post-MI. 10 μ g protein in 100 μ l of PBS injected per mouse. Controls: PBS	Improved LVEF, LVFS, increased number CD4+ T cells observed with hypoxia stimulated EV treatment 7 days post-MI
Mouse CF-derived iPS cells [24]	PEG-precipitation	PKH26 dye, <i>in vitro</i> , 0.5–14 h post-delivery.	2ul EV per 1 well 96 well plate	Male, C57BL/6 mice, 2–3-month-old mice. 45 min I/R via LAD artery ligation.	i.m. injection at the time of MI. 25 μ l EV, dose not reported. Controls: CF-EV and PBS	CF-EV and iPS-EV reduced cardiomyocyte apoptosis compared with PBS treatment. iPS-EV treatment was considerably more effective (~2 fold) than CF-EV.
human biopsy-derived CDC [25]	Commercial precipitation kit ExoQuick	Not assessed	3.5×10^8 CDC-EV or 2×10^8 or Control-EV per 1.5×10^4 cells in eight-chamber slides.	Male, severe combined immunodeficient, 3-month-old mice. Permanent LAD artery ligation.	2 x i.m. injections at time of MI. 40 μ l per injection with a total of 2.8×10^9 CDC-EV and 1.56×10^9 Control-EV. Controls: NHDF-EV, cell culture medium vehicle	15- and 30-days post-MI, LVEF was improved in CDC-EV treated groups compared to controls. CDC-EV treatment decreased scar mass, increased viable mass, and increased infarcted wall thickness compared to NHDF-EV and medium controls.
hucMSC [35]	UC on 30% sucrose/D ₂ O cushions at 100,000 g for 2 h.	Not assessed	200 μ g/mL	Male, Sprague Dawley, 220–250 g rats. Permanent LAD artery ligation.	t.v. injection, timing not reported. 400 μ g protein in 200 μ L PBS. Controls: PBS and EV-depleted conditioned medium	hucMSC-EV treatment reduced scar tissue and apoptosis and increased proliferative staining in hearts of treated rats compared to controls 1-week post-MI. LVFS and LVEF were improved with EV treated 4 weeks post-MI compared to controls.
C57BL/6 mouse-derived ESC [65]	UC on a 30% sucrose-D ₂ O solution 100,000 g for 1 hour	PKH26 dye, <i>in vitro</i> , 16 h post-delivery	Not reported	Male, C57BL/6, 8–12-week-old mice. Permanent LAD artery ligation	2 x i.m. at time of MI. 50 μ g protein in 30 μ l PBS. Controls: MEF-EV and PBS	Improved LVEF, LVFS and ESD were observed with EV-treated mice 1, 2, 3, 4, 6- and 8-weeks post-MI compared to controls. Increased lectin staining (capillary density), reduction apoptosis, increased proliferation was observed in hearts from EV-treated mice 4 weeks post-MI.
C57BL/6 mouse cardiac MSC [26]	PEG-precipitation and NaCl clearance	Not assessed	1 μ g protein 50 μ l medium	C57BL/6 mice, permanent LAD artery ligation.	i.m. injection immediately post-ligation, 50 μ g protein in 30 μ l PBS	LVEF was preserved in the hearts of EV-treated mice 1-month post-MI compared to control mice.
hucMSC, transfected with Akt [31]	UC on 30% sucrose/D ₂ O cushions and 100,000 g for 2 h.	Not assessed	100 μ g/ml	Sprague-Dawley, 220–250 g rats. Permanent LAD artery ligation.	t.v. injection at time of MI. 400 μ g of protein or PBS was. Controls: GFP transfected hucMSC, untransfected hucMSC and PBS	All EV treatments improved LVEF and LVFS 5 weeks post-MI compared to PBS treated rats. Akt-transfected hucMSC-EV had a greater effect than EV control groups. Apoptosis was reduced in hearts of all EV-treated groups compared to PBS treated mice.

(continued on next page)

Table 1 (continued)

EV Source	Isolation method	Uptake assessment	<i>In vitro</i> dose	MI procedure	ROA, <i>in vivo</i> dose and controls	<i>In vivo</i> Outcomes
human right atrial appendage-derived CPC [36]	1. Commercial precipitation kit ExoQuick 2. UC at 100 000 g for 90 min; 3. Commercial precipitation/SEC kits ExoSpin	Dil dye, <i>in vitro</i> , 12 h post-delivery.	0.3 µg, 30 µg and 300 µg protein.	Male, Wistar, 250-300 g, rata. Permanent LAD artery ligation.	3 × i.m. 60 min post-MI 30 µg or 300 µg protein CPC-EV., 300 µg Control-EV in 150 µL Controls: NHDF-EV and PBS	High dose CPC-EV preserved LVEF compared to low dose CPC-EV, F-EV and PBS 7 days post-MI. High and low dose CDC-EV preserved LV diameter compared to NHDF-EV and PBS controls. High dose CPC-EV treatment reduced cardiac fibrosis compared to low dose CPC-EV and controls. High and low dose CPC-EV treatment improved apoptosis and angiogenesis compared to PBS but values for NHDF-EV were not reported.
C57BL/6 mouse bone marrow derived MSC [32]	UC at 110,000 g for 90 min	Dil dye, <i>in vitro</i> , 12 h post-delivery	1 × 10 ¹⁰ EVs per 1 × 10 ⁶ cell.	Male, C57BL/6J, 8-week-old mice. Permanent LAD artery ligation.	t.v. injection Dose not reported Controls: fibroblast (F)-EV (fibroblasts source not specified), cell culture medium vehicle control	MCS-treatment improved LVEF and LVFS compared to controls 4 weeks post-MI. Angiogenesis and fibrosis were improved in MCS-treated hearts compared with controls at 2- and 4-weeks post-MI, respectively.
Rat bone marrow-derived endothelial progenitor cells [27]	Precipitation Exosome Isolation reagent Invitrogen	Celltracker CM-Dil dye, <i>in vitro</i> , 24 hr post-delivery	4.6 × 10 ⁹ EV per well 24-well plate, 7 × 10 ⁴ HUVECs seeded per well	Male, Wistar rats, Permanent LAD artery ligation	5 × i.m. injection at time of MI. 9.33 × 10 ⁹ EV in 100 µl PBS Controls: PBS, shear thinning gel (delivery aid) i.m. injection prior to MI 10 µg protein in 25 µl PBS Controls: PBS	EV-treatment improved LVEF and cardiac output and infarct thickness compared to controls 4 weeks post-MI.
Human and mouse serum (mouse derived EVs used for MI) [28]	Commercial precipitation kit ExoQuick	Not assessed	10 µg/ml	Male, C57BL/6, 8-week-old mice. Permanent LAD artery ligation	10 µg protein in 25 µl PBS Controls: PBS	EV-treatment reduced infarct area and apoptosis compared controls 24 h post-MI.
human ESC-derived cardiovascular progenitors (hESC-Pg) [66]	UC at 99855 g for 16 h	Did dye, <i>in vivo</i> , 24 h post i.m.-delivery	Not assessed	Male, 8-week-old nude mice. Permanent LAD artery ligation	3 × 10 µl i.m. injections (achieved via percutaneous injections under echocardiographic guidance) 2–3 weeks post MI Dose not reported Controls: hESC-Pg, cell culture medium vehicle control	hESC-Pg and hESC-Pg-EV treatment reduced LV ventricular end-systolic, end-diastolic volumes and infarct size compared with vehicle control mice 8–9 weeks post-MI (6 weeks post treatment).

PEG, polyethylene glycol; LAD, left anterior descending; PBS, phosphate buffered saline; ROA, route of administration; I/R, ischaemia reperfusion; i.m., intramyocardial; LVEF, left ventricle ejection fraction; LVFS, left ventricle fractional shortening; CF, cardiac fibroblast; iPS, induced pluripotent stem cell; CDC, cardiosphere-derived cells; NHDF, Normal human dermal fibroblasts; hucMSC, human umbilical cord mesenchymal stem cells; GFP, green fluorescent protein; CPC, cardiac progenitor cells; SEC, size exclusion chromatography; MSC, mesenchymal stem cells; HUVEC, Human umbilical vein endothelial cells; hESC-Pg, human ESC-derived cardiovascular progenitors; UC, ultracentrifugation; t.v., tail vein.

lation methods. EV preparations from these studies likely possess considerable levels of co-isolating protein, particularly serum-derived samples. The effect of which may be more profound in studies that have dosed EV samples using µg of protein; as is the case for BM dendritic cell- [23], BM endothelial progenitor cell- [27], cardiac MSC- [26] and serum- [29] derived EVs. It may be that biologically active EVs are considerably underdosed in these studies and the therapeutic outcomes are understated; however, co-isolated protein is likely to contribute to or mask the effect, if any, of the EVs of interest. Without more thorough characterisation and quantitation of EV preparations these two scenarios cannot be

delineated, and accurate interpretation of efficacy data is not possible.

UC is by far the most commonly used strategy for EV isolation and comparisons between UC and SEC isolation of CPC-derived EVs revealed no significant difference in particle number or whole protein content [30]. Interestingly, EVs isolated through UC have diminished functional capacity compared to EVs isolated using SEC, potentially due to prolonged exposure to high centrifugal forces [30]. Human lung cancer cell-derived EVs also demonstrated shifts in EV size distribution profile in UC- compared to UF-isolated populations, despite the same starting material [20]. Although the dif-

ference in size was speculated to be due to vesicle damaged arising from UC, the underlying mechanism is not clear. Irrespectively, these observations suggest isolation technique can bias EV profile. Small shifts of EV size distribution can be clearly assessed; however, loss of functionality may have downstream effects that are more difficult to characterise. EVs derived from human umbilical cord (huc) MSC [20], embryonic stem cells (ESC), hucMSC transfected with Protein Kinase B (Akt) [31] and BM MSC [32] were all isolated using UC for MI testing. It is possible that these results may underrepresent the full effect EV treatment. Additions to the standard UC protocol have been published, including density gradient and sucrose/ deuterium oxide (D₂O) cushion, additional UC wash and combinatorial approaches with SEC- and precipitation-based methods [21,33,34]. Although these strategies have improved selection and purity [21,33,34], the impact on EV function remains unclear.

Limitations of current dosing methods of EVs for the treatment of MI

Comparisons of studies listed in Table 1 clearly highlight inconsistencies in EV reporting in the various MI models. Overt issues such as omission of dose altogether either for use in preliminary *in vitro* studies or *in vivo* to target MI or insufficient information reported to reproduce the study, e.g. EV volume reported but not the concentration of EV protein and/or numbers of EVs. Two out of the 12 *in vivo* studies [24,32] failed to report the dose of EVs used; out of the remaining studies, variations in isolation method, dosing unit (particle number, μg protein or number of parent cells from which EVs were sourced), animal model (mouse or rat) and route of administration (intramyocardial injection; i.m.; or tail vein; t.v.) render all studies incomparable to each other. Furthermore, often only a single dose is administered, and it is unclear how the dose was selected, whether it is optimal or whether repeat dosing is required for any degree of prolonged efficacy.

Two main dosing strategies are used for EV/MI studies, these are number of particles, as assessed by NTA, and protein concentration. Comparative studies of EV isolation techniques, outlined in the previous section [19,20], very clearly highlight that protein concentration does not correlate with particle number readout. Webber *et al* also demonstrated that spiking EV preparations with bovine serum albumin did not alter the NTA readout [14], again demonstrating the disconnect between the two parameters. Taken together, these findings strongly indicate that protein concentration is not a valid method to determine EV dosing. Although particle number yield can also vary with isolation technique it is more reliable than protein as a surrogate. Reporting both particle number and whole protein content has been suggested as a strategy for assessing sample purity [14]. Routine inclusion of this information may improve standardisation of MI preclinical studies as well as assist in interpretation of efficacy data.

Currently, a validated systematic approach for translating EV-based therapies from *in vitro* to *in vivo* contexts does not exist. Most studies apply an arbitrary increase in dose from *in vitro* to *in vivo* studies [26,27,31,35] and factors such as route of administration, biodistribution and clearance rate are not accounted for. Although these studies demonstrate *in vivo* efficacy, it cannot be determined whether these results represent the full therapeutic capacity of these EV populations. Studies by Barile *et al* assessing the impact of CPC-derived EVs on MI assessed *in vivo* doses of 30 μg and 300 μg of protein observed comparable functional and slightly increased histopathology outcomes in high dose- compared to low dose-EV treated cohorts. Although differences between groups were not proportionate to the fold increase of dose, this study shows that a 10-fold increase in EV dose is not associated with adverse effects [36]. As such, to determine whether an

EV population holds therapeutic potential for the treatment of MI, delivering the maximum feasible dose (dictated by availability and processing capacity of source material and injectable volume) will likely give the best chance of capturing any biological impact EVs may exert. If preliminary findings warrant further investigating, refinement through classical dose-response studies should be undertaken, as well as temporal dosing post-MI to optimise the timing of delivery and identifying the potential need for repeat dosing to sustain any beneficial outcome.

Quantification of EVs

All preclinical EV/MI studies assess particle number using NTA and although a better option than protein quantitation, NTA assessment is not without limitations. Particle number fluctuations have been observed with different equipment types [37] and changes to experimental parameters when using the same equipment [38,39]. Furthermore, separate instruments of the same make and defined experimental parameters can vary greatly in EV characterisation, with assay variation of up to 25% having been reported [40]. Although applying instrument-optimised settings reduces the discrepancies between instruments, this is not routine practice [40]. Therefore, MI studies that have dosed in particle number assessed by NTA (CDC [25] and BM endothelial progenitor cells [27]) may have a dosing error of as much as 25% due to instrument variation; if parameters such as camera height and detection threshold also differ, this error may be greater.

Although tuneable resistive pulse sensing (TPRS) and flow cytometry (FC)-based approaches present alternatives to NTA for measuring EV particle number, they are also prone to many of the same reproducibility issues and lack comparability to direct EV visualisation and assessment via EM [39,41]. With the current resources available, consistent measurements of EV quantity that are reproducible across laboratories is very difficult. Reporting of particle number by high-powered imaging, such as EM, and an additional method, such as NTA or TPRS, is already recommended in the experiential EV guidelines [11] and despite limitations a better strategy does not currently exist.

Impact of Route of Administration on EV Efficacy for Treating MI

One study by Gallet *et al* compared EV route of administration post-MI in a pre-clinical mini pig model and found efficacy to vary depending on delivery method. CDC-derived EVs administered via intracoronary injection decreased microvascular obstruction (MVO) at high dose, but did not reduce infarct size (IS), however, both low- and high-dose EVs reduce IS and MVO after i.m. delivery. These results indicate that i.m. delivery of CDC-derived EVs is more suitable for treating MI [42]. The discrepancy may be due to differences in EV distribution with different dosing routes. Studies outside of the cardiac field also observed that both route of administration (intravenous, intraperitoneal or sub cutaneous injection) and EV source (skeletal muscle cell-, melanoma and dendritic cell-derived EVs) affect biodistribution [43]. It is important to note that these studies were conducted using lipophilic dyes which limit the interpretability of the data, further discussed later. Direct injection of EVs to the site of action (i.m. injection) exerts a greater effect on MI pathophysiology than systemic delivery [42]. As such, it is possible that studies that deliver EVs via t.v. injection; i.e. EVs from BM dendritic cells [23], hucMSC [35], hucMSC transfected with Akt [31] and BM

MSC [32], may only be capturing partial effect of these treatments. Overall, i.m. delivery is likely to capture the full effect of EV impact on MI; however, this is clinically invasive and if systemic delivery is ultimately necessary, the impact of potency and

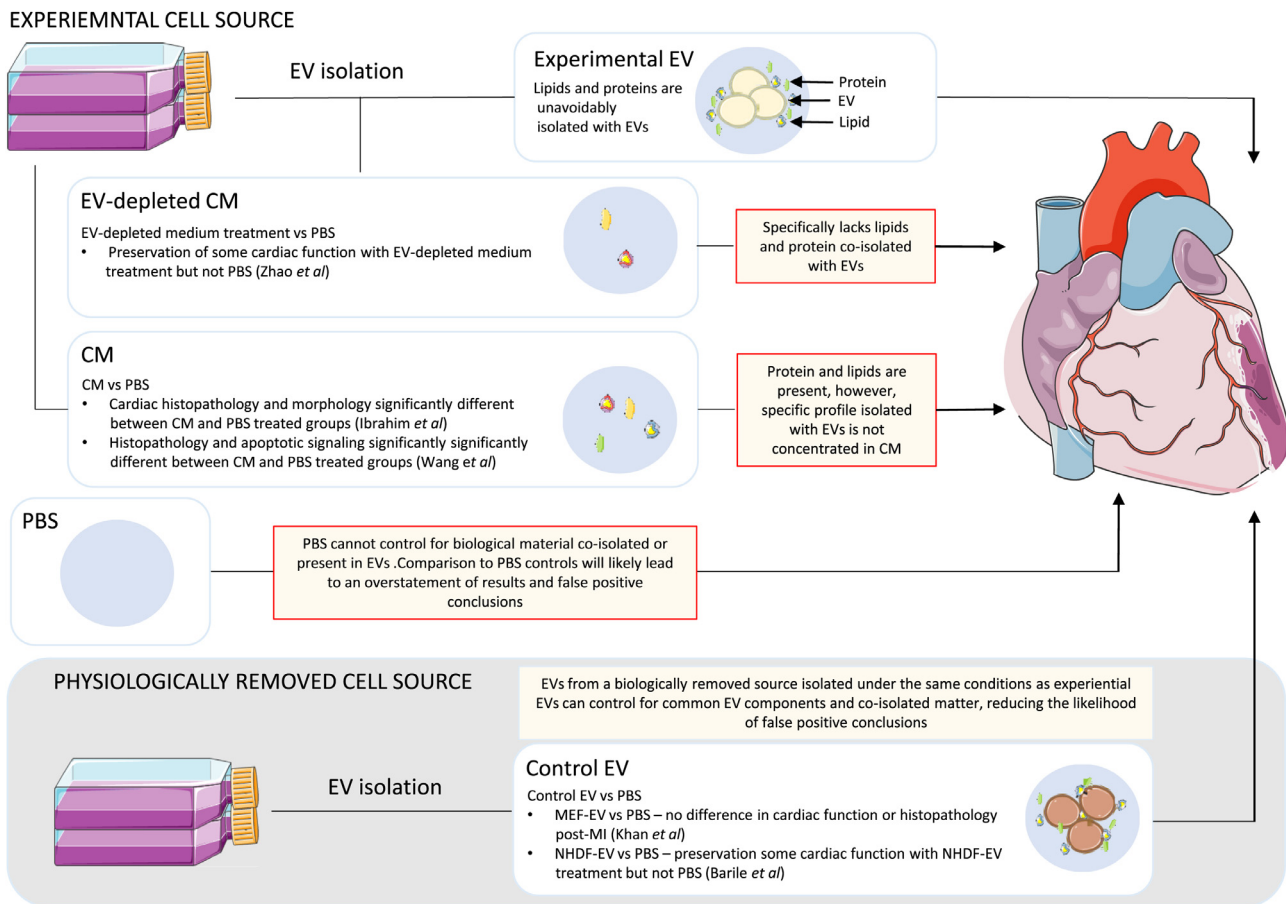


Fig. 2. Negative Controls Used for Extracellular Vesicles (EV)-based Therapies for Myocardial Infarction (MI). Schematic of the negative control utilised for EV/MI efficacy studies to-date and the limitations associated with each strategy. conditioned medium, CM; mouse embryonic fibroblasts, MEF; normal human dermal fibroblasts, NHDF.

ability of the EV population to target the heart should be assessed for each treatment.

Methods for assessing EV uptake in target cells

Lipophilic membrane dyes are by far the most commonly used strategy to assess EV uptake both *in vitro* and *in vivo* studies and were employed exclusively in the MI/EV studies reviewed here. Although many types of dyes have been used for assessing the uptake of EVs (PKH26, PKH67, DiD and CellMask) all work via the same principle whereby they are readily incorporated into lipid structures that integrate and cross the recipient cell membrane [44,45]. Mounting evidence questions their suitability of application in the EV field. Dehghani *et al* demonstrated that incubation of EVs with lipophilic dye (PKH26) increased the size of particles detected via NTA analysis, which may interfere with cell uptake, biodistribution and clearance [46]. Liu *et al* assessed uptake of BM dendritic cell-EVs *in vivo* up to 7 days post-MI [23], bringing into question EV persistence in a biological system. Using a dual-labelled HEK293-EV reporter system, EV signal was observed to decrease in the heart by almost 70% between 30 minutes and 6 hours post-administration [47]. Lipophilic dyes are reported to have an *in vivo* half-life ranging from 5 to >100 days but studies have found persistence of dye as long as 14.5 months after administration in rat hearts [48]. Therefore, EVs are likely degraded and/or recycled *in vivo* while the dyes themselves remained intact and visible in the tissues, yielding inaccurate spatiotemporal information [49]. These comparisons highlight yet an-

other reason lipophilic dyes are not suitable for characterising EV dynamics.

In addition to altered EV properties, Takov and colleagues highlighted the lack of specificity of lipophilic dyes. Through the labelling of SEC-fractionated serum- and conditioned medium-derived EVs, it was shown that dye uptake into target cells did not correspond to EV content and was severely affected by co-isolated lipoprotein and protein content. Dye transfer was also shown to occur in the complete absence of EVs, clearly demonstrated by experiments using lipophilic dye-labelled EV-depleted serum or pure protein samples [49]. This study not only demonstrates the lack of specificity of the lipophilic dye, but given the unavoidable co-isolation of protein with EVs, it also questions the validity of EV uptake studies that have utilised this approach. Alternative techniques are required for testing EV uptake in target cells.

Fusing classical markers of EVs with green fluorescent protein (GFP) has shown efficacy for examining cellular uptake *in vitro* [21,50]. A combined *Gussia* luciferase and biotin reporter system has also been developed that allows for sensitive imaging of *Gussia* luciferase expressing EVs via bioluminescence and biotin can be conjugated to labelled streptavidin for fluorescence-mediated tomography *in vivo*, as well as in organs and biofluids *ex vivo* [47]. Although care must be taken to select the appropriate EV markers in each biological context, these approaches may be useful for accurately assessing EV delivery to target cells. Accurate assessment of EV uptake is essential for answering important biological questions regarding EV biodistribution and half-life which are paramount for clinical progression.

Validity of negative controls for the use of EV treatment of MI

Given the complexity of EV isolation and the unavoidable co-isolation of non-vesicle material, it is clear that appropriate controls must be included in efficacy studies. Negative controls used for MI studies include PBS, medium/vehicle-alone, EV-depleted supernatant and therapeutically inert EV controls. Not all approaches are biologically appropriate and poorly controlled experiments can greatly impact the study validity, outlined in Fig. 2.

These studies highlight the need for the inclusion of control EV-treatments to most accurately interpret the impact of experimental/therapeutic EVs post-MI. In mice, post-MI PBS is clearly not an appropriate control given statistically significant differences have been reported in outcomes when compared to EV control-treated animals. Mice administered conditioned medium as a negative control often yield comparable results to control-EV-treated mice, however, not consistently across all parameters. Although EV-depleted conditioned medium has not been compared to whole conditioned medium, this cannot control for co-isolated protein or EVs present in the culture medium and as such is not an effective negative control. The ideal control is EVs from an alternate (physiologically removed) source, isolated using equivalent protocols as for the experimental EVs. Controls of this nature will account for any therapeutic effect resulting from common EV components and co-isolated material from the EV source and/or medium. These controls are essential for eliminating false-positive results and accurately interpreting efficacy outcomes.

Assessing mode of action of EVs

Thorough characterisation of EV mode of action is paramount for clinical progression of EV-based therapies. An understanding of the cell type and pathways impacted by treatment will allow for optimal application in regard to timing and potential combinatorial approaches. Furthermore, mode of action is necessary for determining target engagement at the desired site of action at the desired time. EV mode of action can focus on a number of processes; the cell types impacted, the EV cargo which exert this effect or the global impact on the recipient tissue. Fig. 3 depicts mechanistic studies conducted in the EV/MI preclinical field.

Although EV cargo assessment has largely focused on RNAs, this does not reflect the diversity of EV cargo. It is important to note that although the miRs that were identified as highly enriched compared to controls (miR146a ~262-fold increase in CDC-derived EVs and the miR-290 family ~10⁴ -fold increase in ESC-derived EVs), only partial recapitulation of the whole EV effect was observed in both contexts. These findings highlight the multifaceted impact of EV uptake on recipient cells and the need for more comprehensive strategies of cargo characterisation. Many other analysis platforms are routinely used in the wider EV field. Recently, mass spectrometry, next-generation sequencing and bioinformatics tools have enabled more detailed proteomic, transcriptomic, lipidomic, metabolomic, glycomic and genomic analyses of EVs, recently reviewed [51]. The EV field is shifting towards systems biology, utilising multiple analysis platforms and convergence of data sets to achieve multi-omic characterisation of EV samples. Such approaches have been successfully used to identify cancer-specific mutations through converged proteomics and genomics [52,53]. The approaches currently taken to examine EV cargo in the EV/MI largely overlook the complex and synergistic nature of whole EV therapies.

Clinical trials of EV-based therapies

Progress in the EV/MI field does not reflect the progress of the EV field as a whole. Although no EV-based therapies have been

approved for patient use across disease indications to-date, many EV-based approaches have entered clinical trials, complete studies include treatments for melanoma [54], colon cancer [55], non-small cell lung cancer [56–58] and chronic kidney disease [59], previously reviewed [60]. However, the most clinically progressed of these studies [59] reflects many of the experimental shortcomings observed in the preclinical EV/MI field. A number of studies [54,56–58] have utilised methods first characterised by Lamparski *et al* in 2002 which describes clinical grade EV isolation from monocyte-derived dendritic cells, recognised as such due to the rapid and reproducible purification method and quality control measures [33]. In brief, the protocol entails ultrafiltration conditioned medium and UC through 30% sucrose/ D₂O cushion to reduce the volume and protein concentration. Importantly, this study also included a purity measure whereby an EV marker was assessed relative to whole protein content. Particle number relative to total protein may offer a comparable readout; however, the use of NTA for assessing EVs was not characterised until 2011 [61]. These studies further highlight the need for scalable isolation methods with the inclusion of purifying steps and measures for the clinical application of EV-base therapies.

Optimal parameters required to accurately interpret findings of EV treatment in MI

As the field stands, major deviations in EV isolation technique, dosing strategy, delivery methods, cell targeting assessment and experimental controls renders findings from many studies incomparable and impossible to interpret (Fig. 4). Uniform criteria are needed so that when EVs are isolated from different sources with different cargo comparisons, resulting data can be more accurately compared for relative efficacy. Based on the reviewed literature we propose the below criteria.

Isolation: currently there is no gold standard for EV isolation; however, preclinical and clinical studies highlight the need for scalable techniques, e.g. UF with subsequent UC, with the inclusion of purifying steps, such as UC wash, Sucrose/D₂O cushion or SEC. Most importantly, it is clear that thorough characterisation of the EV population and contaminates recovered is paramount for more accurately interpreting downstream experiments and improving comparability across the MI studies.

Dosing and quantification: protein amount is not a valid measure for dosing EVs. EVs should be dosed in particle number, assessed as per the minimal experimental requirements for definition of extracellular vesicles and their functions [11]. For proof-of-principle *in vivo* MI studies, applying the maximum feasible dose (limited by availability and processing capacity of source material and injectable volume) will improve likelihood of capturing therapeutic efficacy. Inclusion of purification steps during isolation and routine reporting of protein amount and particle number will reduce co-isolated non-vesicle material and limit non-specific effects at EV higher dosing. If EVs are determined to warrant further investigation, studies can be refined classical dose-response, optimal timing and repeat dosing experiments.

Route of administration: i.m. delivery appears to be the most appropriate for proof-of-principle efficacy studies of MI. Systemic administration of EVs may diminish efficacy and lessen/preclude uptake into target tissue. Rate of turnover of EVs in serum versus target tissue needs to be evaluated and each approach should be tailored for the specific EV population. Technologies for tagging and targeting therapeutic EVs are emerging which will be of use here in tracking delivery and persistence in the target tissue.

Uptake and biodistribution: lipophilic dyes are not appropriate for assessing EV dynamics. Integrated reporters (e.g. green fluorescent protein) in a gene known to be enriched in the EV population of interest should be utilised for assessments of cell targeting and

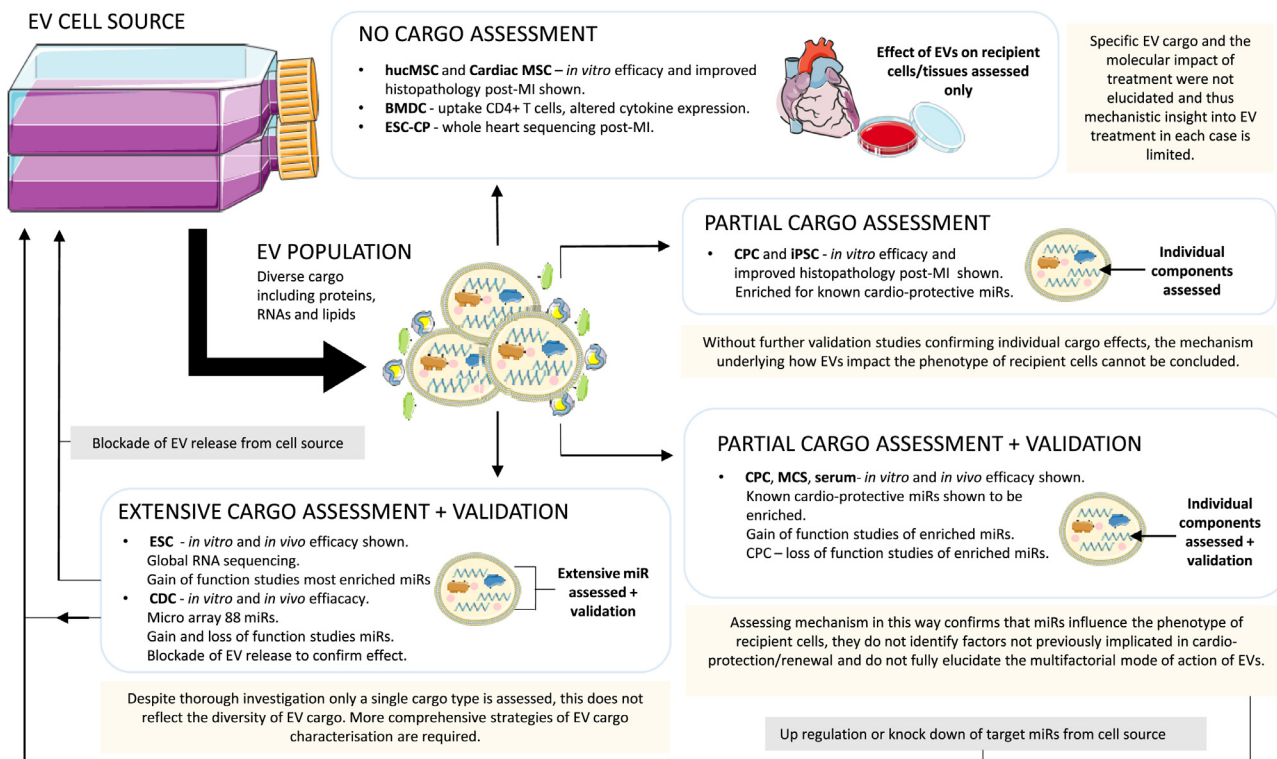


Fig. 3. Mode of Action of Extracellular Vesicles (EV) Investigated for the Treatment of Myocardial Infarction (MI). Schematic of the mechanistic studies undertaken in the preclinical EV/MI field. Micros RNAs, miRs; the following denote EV sources; human umbilical cord, huc; mesenchymal stem cells, MSC; bone marrow-derived dendritic cells, BMDC; embryonic stem cell-derived cardiac progenitors, ESC-CP; cardiac progenitor cells, CPC; induced pluripotent stem cells, iPSC; cardiosphere-derived cells, CDC; embryonic stem cell, ESC.

biodistribution. Ideally alternative methods with integrated minimally invasive/disruptive means for detection need to be developed.

Negative controls: vehicle (PBS) or EV-depleted medium are inadequate controls. A therapeutically inert (biologically removed) EV control, isolated under the same conditions as experimental EVs is most appropriate for efficacy studies. Controls of this nature will largely account for co-isolated proteins and common EV components allowing for better interpretation of experimental EV data.

Mode of action: The approaches currently taken to examine EV cargo in the EV/MI largely overlook the complex and synergistic nature of whole EV therapies. A combination of unbiased characterisation, such as proteomic and transcriptomic analyses, should be applied for cargo characterisation. Loss and gain of function studies for enriched cargo will assist in validating and efficacy and mode-of-action. Given the multifaceted nature of EV functions, multiple analysis platforms and convergence of data sets will be necessary for a more comprehensive understanding of whole EV effects on recipient cells.

Adherence of preclinical EV/MI studies to the above framework may assist in more accurate interpretation of preclinical data and comparability between studies. Such refinements may improve our understanding of EV-based therapies and facilitate translation into clinical trials.

Future directions and considerations for translation of EV-based therapies to clinical trials

Clinical application of EV-based therapies will require stringent good manufacturing practice (GMP)-compliant experimental protocols, regarding both EV source and EV product to be administered to patients. The *International Society for Extracellular Vesicles* (ISEV) recently published an in depth review of the considerations and

legislation pertaining to the translation of EV-based therapies to clinical trials [62]. EVs are unique in the fact the donor source and the EV product must both meet clinical standards of consent, reproducibility and sterility [62,63]. Andriolo *et al* published protocols for the large scale production of CPC-derived EVs for future clinical application in AMI in which these standards are met [64]. Authors were able to reproducibly culture patient-derived CPC cells in xeno-free conditions, producing large volumes (up to 8L) of conditioned medium for EV isolation. Importantly both CPC parent cells and EV samples were subject to extensive quality controls (QC) screening panels ensuring medical grade sterility. Assessments of storage conditions found that storage of EVs at -80°C did not diminish functional capacity. Furthermore, a standardised patient-derived CPC bank was established to allow for reproducible re-expansion and isolation of EVs for application in clinical trials, subject to QC checks. Comparable strategies could be applicable for other primary cell types to facilitate translation to clinical trials. Although immortalised cells lines are not appropriate for transplantation, if it can be demonstrated that the immortalising genes are not captured in EVs then these cell lines may offer highly scalable and homogenous sources of EVs [62]. Results of numerous phase I clinical studies have demonstrated the feasibility and safety of autologous EV-based therapeutics [54–58], only one has investigated allogenic EVs [59]. Nassar *et al* found that MSC-derived EV administered to patients with chronic kidney disease were safe and well tolerated and improved kidney function through reductions in inflammation [59]; although promising, allogenic capability cannot be assumed for all EV sources. Together these findings highlight the importance of comprehensive QC screening for both EV cell source as well as EV end product. The feasibility of large-scale production, long-term storage and potential for allogenic application suggest EV-based therapies could offer an off-the-shelf solution for AMI.

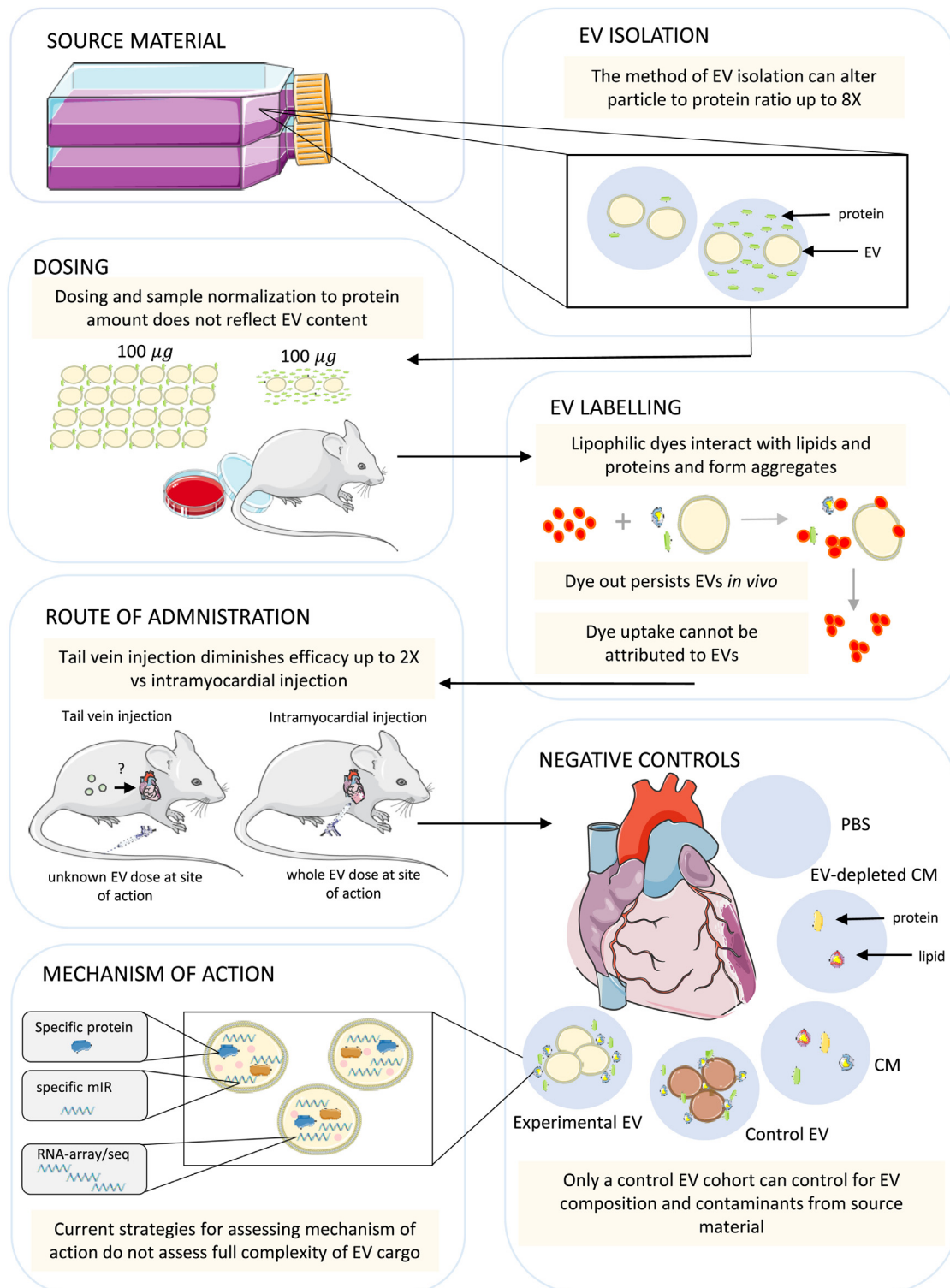


Fig. 4. Overview of the current experimental limitations for the preclinical assessment of extracellular vesicles (EVs) for myocardial infarction (MI). Different EV isolation methods can yield samples with up to an 8-fold difference in protein content relative to particle number from the same source material (due to co-isolating contaminating proteins). The majority of EV/MI studies are dosed in protein content (opposed to particle number), this can result in an 8-fold difference in particle number in EV samples normalised to the same protein amount. Lipophilic dyes are the only tool used to label EVs in the preclinical MI field. These dyes are not specific to EVs and also interact with protein and lipids and can form dye aggregates, therefore the presence of dyes cannot be attributed to EV uptake in cells. Lipophilic dyes can also out persist EVs *in vivo* leading to false conclusions regarding spatial and temporal distribution profiles. EVs delivered via intramyocardial injection compared to systemic delivery can have a 2-fold greater impact of efficacy outcomes of MI, preclinically. Numerous negative controls have been used across studies including; PBS, EV-depleted conditioned medium (CM), CM and therapeutically inert EV populations. With the exception of inert EV controls, negative controls do not account for co-isolated contaminants or common EV components and comparison to experiential EVs may overstate results. The majority of mechanistic studies to date have assessed previously characterised markers of cardiac regeneration, only two studies applied broader analysis via RNA-seq and PCR array. The complexity of EV cargo is not assessed in these studies and findings may not be sufficient for clinical progression. Overall each of these steps have the potential to introduce fold changes in efficacy outcomes and various combination of these approaches has rendered all studies in the EV/MI field completely incomparable. In many case poor results are difficult to interpret and impossible to contribute to EV effect alone.

Table 2
Limitations for clinical progression of extracellular vesicles.

Clinical criteria	EV current status
Safety and scalability	Scalability and GMP of source cells and EV products have been demonstrated however application to clinical trials has not yet commenced. Extensive quality control checks are included in guidelines to ensure safety standards are met. Purity of the EV preparations must also become standard to ensure accurate efficacy readouts and dosing but also to limit the inclusion of unknown contaminants and ensuring a defined solution is delivered to patients.
Efficacy/dose optimisation	Accurate efficacy studies are dependent on accurate quantitation of EVs. Although best practice standards are in place, these are still hampered by comparability between samples assessed at different sites. Improved methods for EV quantitation are paramount.
Biodistribution	Accurate labelling of EVs in a manner that is specific and does not interfere with EV dynamics or function is essential for assessing <i>in vivo</i> spatial distribution and persistence/half-life.
Bioavailability at site of action	Ability of EVs to reach the site of action at the desired time is similarly dependent on accurate labelling with <i>in vivo</i> , real-time imaging capacity.
Target engagement/MOA	Meaningful target engagement of EV-based therapies must be ascertained to define mechanism of action. The development of new strategies to capture these interactions between EV cargo and recipient cells <i>in vivo</i> will likely require the development of new technologies.

GMP, Good manufacturing practice; EV, Extracellular vesicle, mechanism of action; MOA.

Evidence from both within the cardiac field and across numerous others has identified EVs as promising therapeutic agents with a high clinical potential. However, EV-based therapies are currently limited by a lack of understanding of precise target engagement and the inability to accurately and reproducibly quantify EV potency or measure essential biodistribution and pharmacokinetic information, reliant on specific labelling (Table 2). Although some of these hurdles may be overcome with existing methodologies, such as use of integrated labelling systems for assessing *in vivo* EV dynamics, others will require a better understanding of the underlying biology and the development of new technologies. In particular, the development of more accurate and robust methods for measuring EV number, assessment of sample purity and defining what constitutes meaningful target engagement in an EV context. Adapting experimentally and addressing the dearth in technology will be paramount for the transition of EV-based therapies into the clinic.

Concluding remarks

Although a therapeutically promising field, preclinical EV/MI research has suffered from experimental approaches that are not fully standardised or validated. EV isolation, quantification, dosing, uptake assessment and inappropriate controls are all areas in need of further refinement. Although some of these shortcomings can be easily remedied by existing techniques, others require the development of new platforms and strategies. Fundamental improvements in EV study design, are required to provide confidence in interpretation of efficacy and to ensure reproducibility and comparability across preclinical MI studies.

Author contributions

PRR conceived the idea for the review topic. TK, AJR and PRR discussed content. TK drafted the text in full. AJR and PRR edited successive revised versions.

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