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REVIEW

Clinical update

Roles of exosomes in cardioprotection

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Exosomes are extracellular vesicles of endosomal origin which have emerged as key mediators of intercellular communication. All major cardiac cell types—including cardiomyocytes, endothelial cells, and fibroblasts—release exosomes that modulate cellular functions. Exosomes released from human cardiac progenitor cells (CPCs) are cardioprotective and improve cardiac function after myocardial infarction to an extent comparable with that achieved by their parent cells. Cardiac progenitor cell-derived exosomes are enriched in cardioprotective microRNAs, particularly miR-146a-3p. Circulating exosomes mediate remote ischaemic preconditioning. Moreover, they currently are being investigated as diagnostic markers. The discovery that cell-derived extracellular signalling organelles mediate the paracrine effects of stem cells suggests that cell-free strategies could supplant cell transplantation. This review discusses emerging roles of exosomes in cardiovascular physiology, with a focus on cardioprotective activities of CPC-derived exosomes.

Keywords Cardioprotection • Exosomes • Progenitor cells

Introduction

Ischaemic heart disease is associated with loss of cardiomyocytes, ultimately leading to pump failure in a substantial proportion of patients. Over the past 15 years, cell transplantation has been evaluated as an approach for the disease.[1] While an early study in mice reported that haematopoietic stem cells injected in infarcted hearts differentiated into cardiomyocytes and enhanced cardiac function,[2] subsequent studies failed to confirm these findings.[3] Despite this controversial experimental foundation, clinical trials of bone marrow cell transplantation in patients after acute myocardial infarction (MI) were initiated quickly, demonstrating feasibility and suggestive of possible benefits.[4] However, the conclusion that bone marrow cell transplantation improves function has been questioned.[5] Recently, mesenchymal stem cells (MSCs) and cardiac progenitor cells (CPCs) have been evaluated as alternate cellular sources. In a phase-I clinical trial in patients after acute MI, intramyocardial injection of autologous cardiosphere-derived cells (CDCs), obtained from spontaneously forming spherical aggregates of CPCs *in vitro*,[6] was safe and provided promising results.[7] Although by definition CPCs can produce new cardiomyocytes, the actual mechanism of benefit *in vivo* is indirect: transplanted cells are not found in the heart at late time points post-delivery and, after injection of human CDCs in infarcted mouse hearts, ~90% of newly formed cardiomyocytes and endothelial cells (ECs) are of endogenous origin.[8] Likewise, c-kit+CPCs generate vanishingly few mature cardiomyocytes but stimulate the proliferation of endogenous cells in the heart, which persists for at least 1 year post-injury.[9] Thus, exogenous cell transplantation augments endogenous repair via paracrine factors. While these factors were first thought to be small proteins and cytokines,[8,10] we have recently shown that extracellular vesicles (EVs) figure prominently in the bioactivity of human CPCs.[11,12] Injected CDC-derived EVs in infarcted mouse hearts reproduced the benefit of CDC administration, and blockade of exosome secretion nullified CDC bioactivity. Thus, EV-based cell-free strategies could supplant cell transplantation. This review discusses emerging roles of EVs in cardiovascular physiology, with a focus on EV-mediated cardioprotection. Potential roles of exosomes as diagnostic markers will also be addressed. To contextualize functions of EVs in cardiac physiology within the broader field of vesicle biology, EV biogenesis is summarized in the following section.

Extracellular vesicle subpopulations

Cells secrete EVs of various sizes and intracellular origins, including exosomes, microvesicles, and apoptosomes. Extracellular vesicle populations were traditionally categorized by size, but recognition of differential biogenesis has become an additional qualifier of

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identity. Exosomes (diameter range: 30–150 nm) are EVs of endosomal origin. Microvesicles (diameter range: 50–1000 nm) arise by direct budding from plasma membranes of injured or transformed cells. Various stimuli—including intracellular calcium concentration and lipopolysaccharide (LPS)—trigger microvesicle shedding.[13] Apoptosomes (diameter range: 50–5000 nm) originate as fragments of cells undergoing programmed death.

Proteomic comparisons have identified novel markers for the characterisation of heterogeneous EV populations.[14] Extracellular vesicles were subdivided into large, medium-sized, and small vesicles pelleting at low (2000 × *g*), intermediate (10 000 × *g*), and high (100 000 × *g*) sedimentation speeds, respectively, the last being the fraction classically considered as containing exosomes. Small (<200 nm) EV preparations contained both exosomal and non-exosomal subpopulations, which were further subdivided into: (i) *bona fide* exosomes co-enriched in exosome (CD63, CD81, CD9) and endosome markers (syntenin-1, tumour susceptibility gene-101); (ii) small EVs devoid of CD63/CD81 but enriched in CD9; (iii) small EVs devoid of CD63/CD9/CD81; (iv) small EVs enriched in extracellular matrix proteins or serum-derived factors. For now, it is important to recognize that the nomenclature tends to be used rather loosely, and that it may be difficult in practice to distinguish exosomes from other EVs.

Exosome biogenesis

Secretion of exosomes has been demonstrated across eukaryotes from amoeboid protists to fungi, plants, and animals. Exosomes originate from multivesicular bodies (MVBs) that arise from invaginations of the plasma membrane fusing to molecular cargoes sorted into the endoplasmic reticulum and processed in the Golgi complex. When MVBs fuse to the plasma membrane, exosomes are released into the extracellular space; however, some of the MVBs merge with lysosomes for degradation, rather than being secreted (*Figure 1*). Exosomes released from different cell types transport distinct lipid, protein and nucleic acid cargoes. They also contain cytokines, pathogen-associated and damage-associated molecular patterns, and autoantigens. Endosomal-sorting complex required for transport is a central component of the molecular machinery of exosome formation. Protein and RNA sorting into exosomes are highly

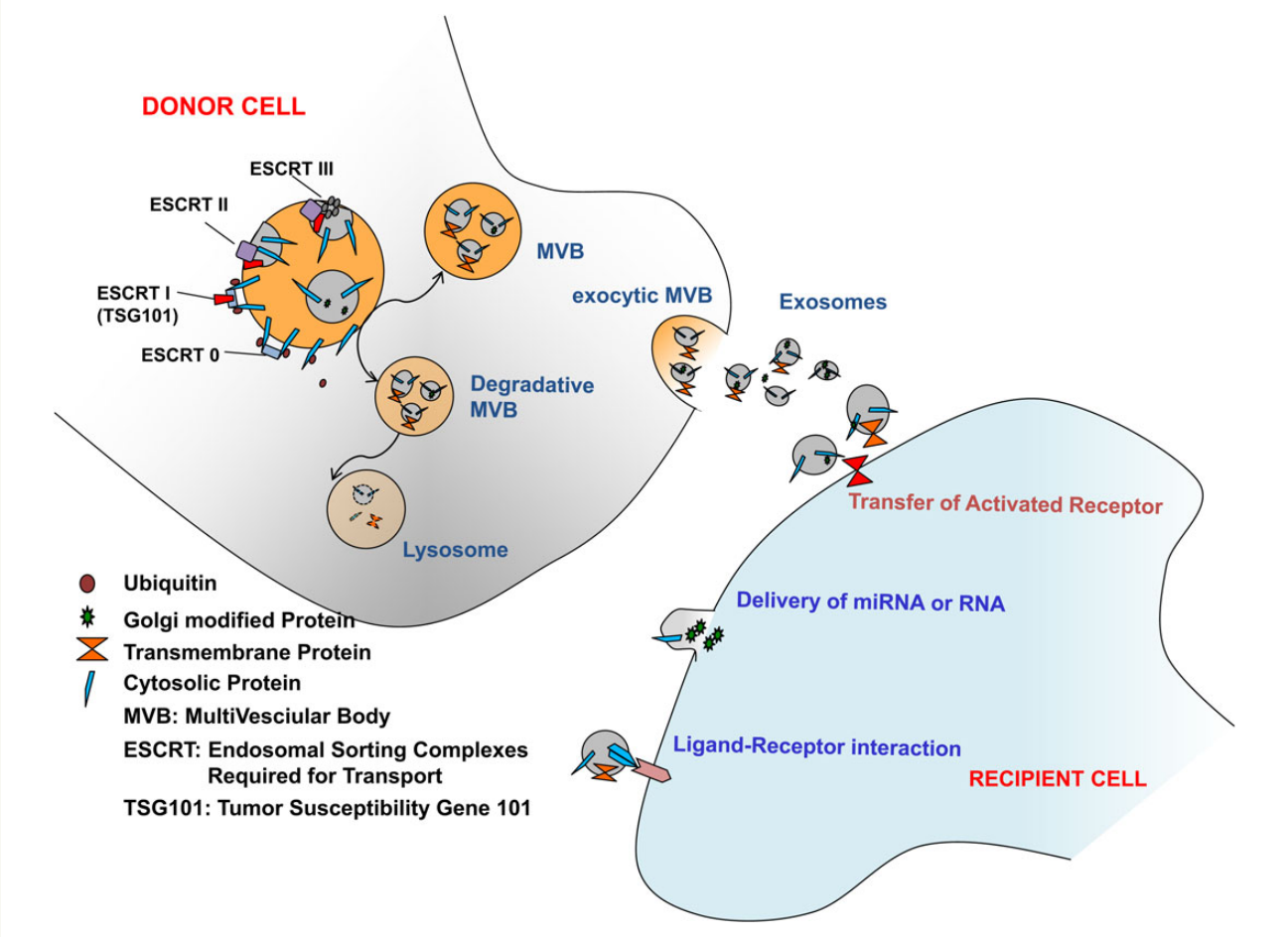

Figure 1 Exosome biogenesis. Endosomes originate by internal budding of plasma membranes. They contain proteins from plasma membranes and from the Golgi complex, and nucleic acids. Multivesicular bodies containing late endosomes fuse with the plasma membrane or undergo lysosomal degradation. The endosomal-sorting complex required for transport facilitates protein sorting into exosomes. Exosomes are internalized by recipient cells, transfer activated receptors to cell surfaces, or bind to surface receptors to activate signalling pathways.

regulated processes that allow cells to release exosomes with varying characteristics depending on the molecular signals that induce their production. Protein sorting mechanisms involve interactions between CD63, other trans-membrane proteins, the multivalent adaptor syntenin, and Alix.[15] Specific motifs present in certain microRNAs (miRs) facilitate their incorporation into exosomes through binding to chaperon proteins.[16] Post-transcriptional modifications may regulate miR sorting.[17] Exosome-delivered mRNA can be translated into proteins, and exosome-delivered miRs can target mRNA expression in recipient cells. The online compendium ExoCarta (http://www.exocarta.org) provides an updated list of the biomolecules found in exosomes. Examples of cardiovascular effects of EVs are shown in *Table 1*.

Exosome-based diagnostic markers

The first area of clinical translation of EVs has been in diagnostics (*Figure 2*). Proteins and RNAs specific to their cells of origin are incorporated into EVs and released into biofluids, where their contents can be measured as biomarkers (*Figure 3*). Although several studies evaluated serum miRs as cardiovascular markers, only rarely was the location of these miRs inside EVs addressed. Levels of miR-1 and miR-133a were increased in sera from patients with acute MI, unstable angina pectoris, or Takotsubo cardiomyopathy.[18] Dead cardiomyocytes were shown to release these miRs via exosomes *in vitro*. Measurements of concentration gradients across the coronary circulation demonstrated the myocardial release of cardiomyocyte-enriched miR-133a and miR-499 in patients with troponin-positive acute coronary syndromes.[19] In patients with acute MI, plasma levels of p53-responsive miRs (miR-192, miR-194, miR-34a) inside EVs were associated with development of heart failure.[20] In patients with stable coronary artery disease, expression of miR-126 and miR-199a in circulating microvesicles, but not freely circulating miRs, predicted the occurrence of cardiovascular events.[21] These findings exemplify possible roles of EVs as diagnostic markers. However, the accuracy of a given EV-based test mandates careful validation.

Cardiomyocyte-derived exosomes

The first study on EV secretion by cardiomyocytes reported the release of the cytoprotective heat shock proteins (HSPs) 70 and 90, along with HSP60, a putative 'danger signal', via exosomes in rat cardiomyocytes.[22] Glucose deprivation caused the release of exosomes enriched in functional glucose transporters and glycolytic enzymes from rat cardiomyocytes. These exosomes were internalized by ECs where they increased glucose uptake and glycolytic activity. Hyperglycaemia altered cardiomyocyte-derived exosomes in a model of diabetes-associated cardiomyopathy.[23] After externally imposed cellular stretch, cardiomyocytes released exosomes enriched in functional angiotensin II type-1 receptors (AT1Rs).[24] Administration of AT1R-enriched exosomes restored blood pressure responsiveness to angiotensin II in AT1R-KO mice. These findings introduce the intriguing concept that endogenous surface proteins on EVs may effect tissue-specific targeting.

Table 1 Cardiovascular effects of exosomes

Releasing cell type	Molecular mediator(s)	Stimulus	Recipient cell	Biological effect	Ref.
Therapeutic effects					
Rat CM	HSP70, HSP90	Hypoxia	CM	Cytoprotection	22
EC	miR-214	–	EC	Migration, angiogenesis	26
MSC	miR-22	Ischaemic preconditioning	CM	Cardioprotection	32
Human CDC	miR-146a	–	CM, fibroblast	Cardioprotection, anti-fibrosis	11
Human CPC	miR-210, miR-132	–	CM, EC	Cardioprotection, angiogenesis	12
Mouse CPC	miR-292, miR-210, miR-103, miR-17	Hypoxia	EC, fibroblast	Angiogenesis, anti-fibrosis	34
ND (plasma Exo)	HSP70	RIC	CM	Cardioprotection	35
Mouse cardiac cells	miR-144	RIC	CM	Cardioprotection	38
Pathogenic effects					
Mouse CM	Angiotensin II R1	Cardiac pressure overload	CM, EC, VSMC	Angiotensin II responsiveness	24
Human microvasc. EC	ICAM-1	TNF-α	EC	Vascular inflammation	25
Mouse EC	miR-146a	16-kDa N-terminal prolactin fragment	CM	Depressed CM function	27
Cardiac fibroblast	miR-21-3p/miR-21*	Angiotensin II	CM	CM hypertrophy	29
Platelet	Superoxide, NO, peroxynitrite	LPS, NO	EC	Apoptosis	42
CM	miR-320	Hyperglycaemia	EC	Anti-angiogenesis	23

Examples of beneficial or pathogenic effects of exosomes are shown (CM, cardiomyocyte; EC, endothelial cell; HSP, heat shock protein; MSC, mesenchymal stem cell; NO, nitric oxide; VSMC, vascular smooth muscle cell; RIC, remote ischemic preconditioning).

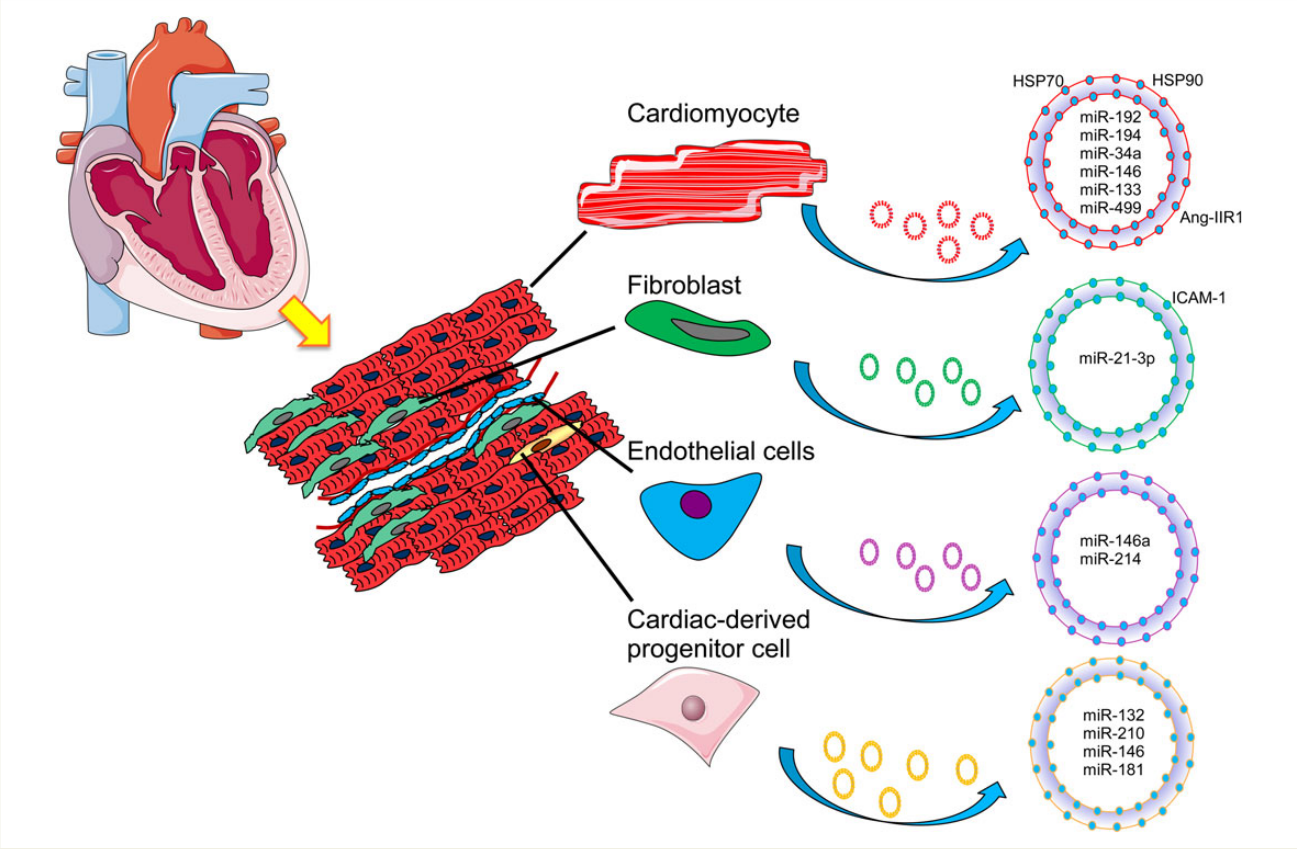

Figure 2 Exosome release by different cardiac cell types. Cardiomyocytes, ECs, cardiac fibroblasts, and cardiac progenitor cells release exosomes. Examples of exosome-enriched miRs are shown (see *Table 1*).

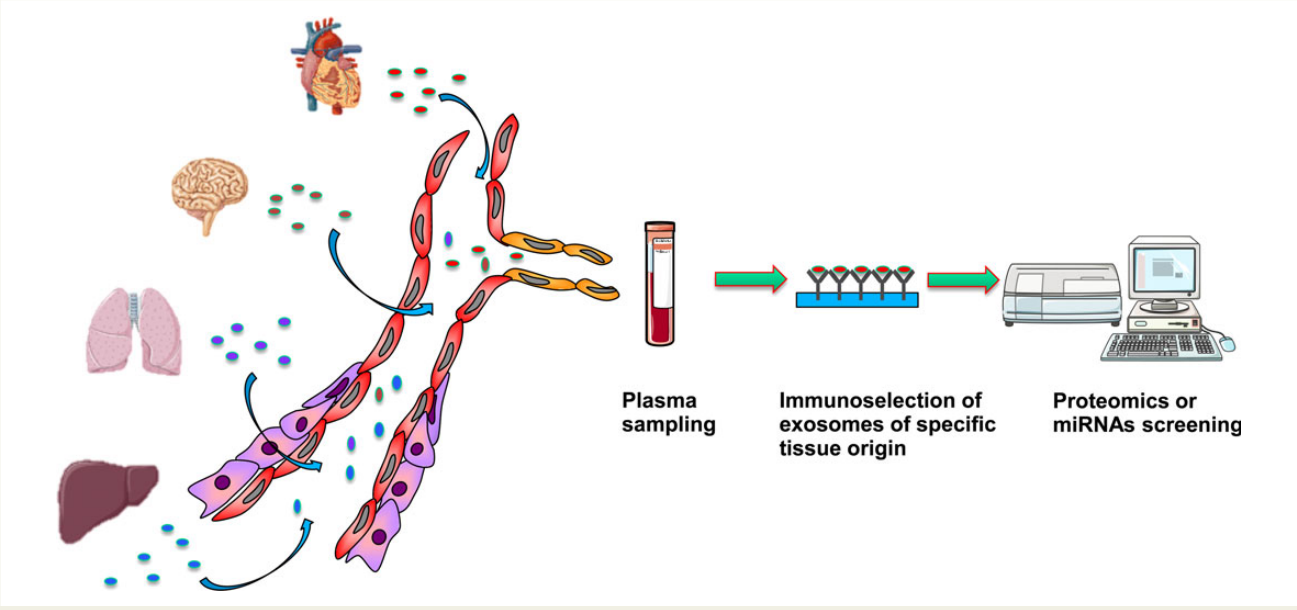

Figure 3 Exosomes as diagnostic markers. Organs release exosomes into the circulation. Exosomes originating from different organs can be immunoselected for proteomics and miR profiling.

Endothelial cell-derived exosomes

Vascular ECs secrete vesicles that exchange biological messages with cardiomyocytes, smooth muscle cells, and fibroblasts. Tumour necrosis factor (TNF)-α-treated ECs released exosomes expressing increased levels of intercellular adhesion protein (ICAM)-1.[25] In another study, EC-derived exosomes stimulated angiogenesis in recipient ECs via miR-214 transfer.[26] In a model of post-partum cardiomyopathy, the 16-kDa N-terminal prolactin fragment induced the release of miR-146a-enriched exosomes from ECs.[27] These exosomes were internalized by cardiomyocytes where they increased miR-146a levels, downregulated miR-146a target genes, and depressed contractile function. Blocking miR-146a using locked nucleic acids or antago-miRs attenuated a post-partum cardiomyopathy like phenotype associated with increased miR-146a expression in cardiomyocyte-restricted Stat3-KO mice. These findings exemplify pleiotropic effects of EC-derived exosomes on recipient ECs and cardiomyocytes.

Cardiac fibroblast-derived exosomes

Various forms of stress in the heart—including ischaemia, hypertension, and valvular dysfunction—induce hypertrophic cellular responses mediated by cross-talk among cardiomyocytes, fibroblasts, ECs, and inflammatory cells via EVs. In response to angiotensin II, cardiac fibroblasts secreted exosomes that stimulated angiotensin II production and its receptor expression in cardiomyocytes and promoted myocyte hypertrophy.[28] Exosomes released from cardiac fibroblasts contained high levels of miR-21-3p/miR-21* (one of the 'star' miR passenger strands that normally undergo intracellular degradation), which promoted cardiomyocyte hypertrophy.[29]

Stem cell-derived exosomes

Exosomes released from bone marrow-derived CD34+ cells reproduced the angiogenic activity of their parent cells in experimental models.[30] Mesenchymal stem cell-derived exosomes increased ATP levels, reduced oxidative stress, and activated the PI3K/Akt pathway to enhance cardiomyocyte viability after ischaemia/reperfusion (I/R).[31] Ischaemic preconditioning potentiated MSC-mediated cardioprotection via miR-22-enriched exosomes.[32] These findings suggest that the benefits of MSCs may be mediated by the exosomes they secrete.

Cardiac progenitor cell-derived exosomes

Recent studies from our laboratories showed that exosomes released from human CDCs,[11] or from CPCs not subjected to cardiosphere formation *in vitro*,[12] were cardioprotective. They inhibited stress-induced cardiomyocyte apoptosis, induced cardiomyocyte proliferation, and stimulated angiogenesis compared with dermal fibroblast-derived exosomes *in vitro*. *In vivo*, CDC-derived exosomes injected into the infarct border zone reduced scar, increased viable mass and infarcted wall thickness, and improved global heart function in mice compared with fibroblast-derived exosomes or control media. Pre-treatment of CDCs with the exosome biosynthesis inhibitor GW4869 abolished CDC-mediated cardioprotection *in vivo*. Cardiac progenitor cell-derived exosomes were similarly cardioprotective *in vitro*. *In vivo*, they reduced the number of apoptotic cardiomyocytes in the infarct border zone and scar while enhancing global heart function after permanent coronary artery occlusion in rats.[12] The two studies independently identified miR-146a as the most highly enriched miR in both CDC-exosomes and CPC-exosomes relative to fibroblast-exosomes. A miR-146a mimic inhibited oxidant stress-induced cell death in rat cardiomyocytes.[11] miR-146a-KO mice had larger infarct areas compared with wild-type mice of the same strain; in such mice, a miR-146a mimic injected at the time of MI 'rescued' increased infarct size. miR-146a-deficient exosomes, derived from CDCs transfected with a miR-146a hairpin inhibitor, were less protective against oxidant stress than control CDC exosomes. Thus, CDC-derived exosomes mediate cardioprotection, at least in part via miR-146a transfer.

Exosomes released from CDCs altered the secretory profile of dermal fibroblasts.[33] Priming fibroblasts with CDC-released exosomes caused them to secrete much higher levels of stromal cell-derived factor-1 and vascular endothelial growth factor, and dramatically changed miR profiles of fibroblast-secreted EVs. Intramyocardial injection of CDC-exosome-primed fibroblasts, but not unprimed fibroblasts, increased pump function, and vessel density while reducing scar mass in rat hearts after chronic MI. Thus, CDC-derived exosomes converted inert fibroblasts to therapeutically active cells.

Hypoxia stimulated exosome release from mouse CPCs and modified their molecular content.[34] Expression of pro-angiogenic genes, anti-fibrotic genes, and a cluster of miRs was upregulated in hypoxic CPC-exosomes. These reduced myocardial fibrosis and enhanced pump function after I/R *in vivo*.

These results indicate that CPC-derived exosomes inhibit cardiomyocyte death during ischaemia and I/R. Limited data on their roles in cardiac repair and regeneration are available. Angiogenic and anti-fibrotic activities of CPC-derived exosomes likely contribute to tissue repair. We have shown that CDC-derived exosomes injected at 21 days post-MI, a time point at which myocardial scar is well-established, still reduce scar, and increase viable myocardial mass in mice.[11] Moreover, injected CDC-exosome-primed fibroblasts increased pump function and reduced scar mass in rat hearts after chronic MI.[33] These findings support the notion that exosomes mediate therapeutic regeneration.

Circulating exosomes and preconditioning

The exosome-rich fraction of plasma protected against myocardial I/R injury *in vivo*.[35] HSP70 expressed on the exosome surface bound to toll-like receptor-4 expressed on the cardiomyocyte surface, activating pro-survival pathways in cardiomyocytes.

Remote ischaemic preconditioning (RIPC) of the heart is induced by brief ischaemic insults inflicted on a remote organ or myocardial region before sustained myocardial ischaemia.[36] Coronary perfusates of isolated rat hearts exposed to three cycles of I/R were enriched in EVs compared with hearts exposed to continuous aerobic perfusion, and the perfusates from I/R donor hearts reduced infarct size to an

extent comparable with ischaemic preconditioning. The protective effect was abrogated by depleting the perfusates of EVs.[37] Coronary RIPC upregulated miR-144 precursor in exosomes and miR-144 in myocardium.[38] Systemic miR-144 administration reduced infarct size to an extent comparable with RIPC, whereas specific miR-144 antisense oligonucleotides abolished this effect of RIPC.

Cardiosphere-derived cells administered with 30 min delay after reperfusion in pigs reduced infarct size, a phenomenon termed 'cellular post-conditioning' to distinguish it from conventional ischaemic post-conditioning, which wanes earlier.[39] Macrophages mediated the benefits of CDCs in a rat model of reperfused MI, via soluble factors, which turned out to be exosomes.[40,41]

Pathogenic roles of extracellular vesicles

The complexity of EV-mediated effects is illustrated by the observations that CPC-derived, miR-146a-enriched exosomes were cardioprotective,[11,12] whereas EC-derived, miR-146a-enriched exosomes promoted pregnancy-associated cardiomyopathy.[27] Platelet-derived exosomes mediated myocardial inflammation in an LPS-induced model of sepsis.[42] They induced EC apoptosis by generating superoxide, NO, and peroxynitrite. Circulating miR-320-enriched exosomes inhibited EC proliferation and migration in diabetic rats, suggesting a role in diabetic microangiopathy.[23] Exosomes also play important roles in communication between blood cells and vascular tissues in atherogenesis.[43]

Exosomes and soluble factors as paracrine effectors

The secretome of bone marrow cells has been analysed.[10] Soluble factors released by these cells were traditionally believed to account for their paracrine activities. However, we have shown that exosome depletion abolishes cardioprotection mediated by CPC-conditioned media.[12] Likewise, exosome depletion abrogated RIPC mediated by coronary perfusates of donor hearts exposed to transient I/R.[37] These findings identify exosomes as the active component of the paracrine secretion by CPCs, and by cardiac cells in RIPC. Exosomes protect the sequestered proteins and RNA from degradation, facilitating their delivery to recipient cells. Exosomal protein and miR contents differ from the secretomes (and contents)

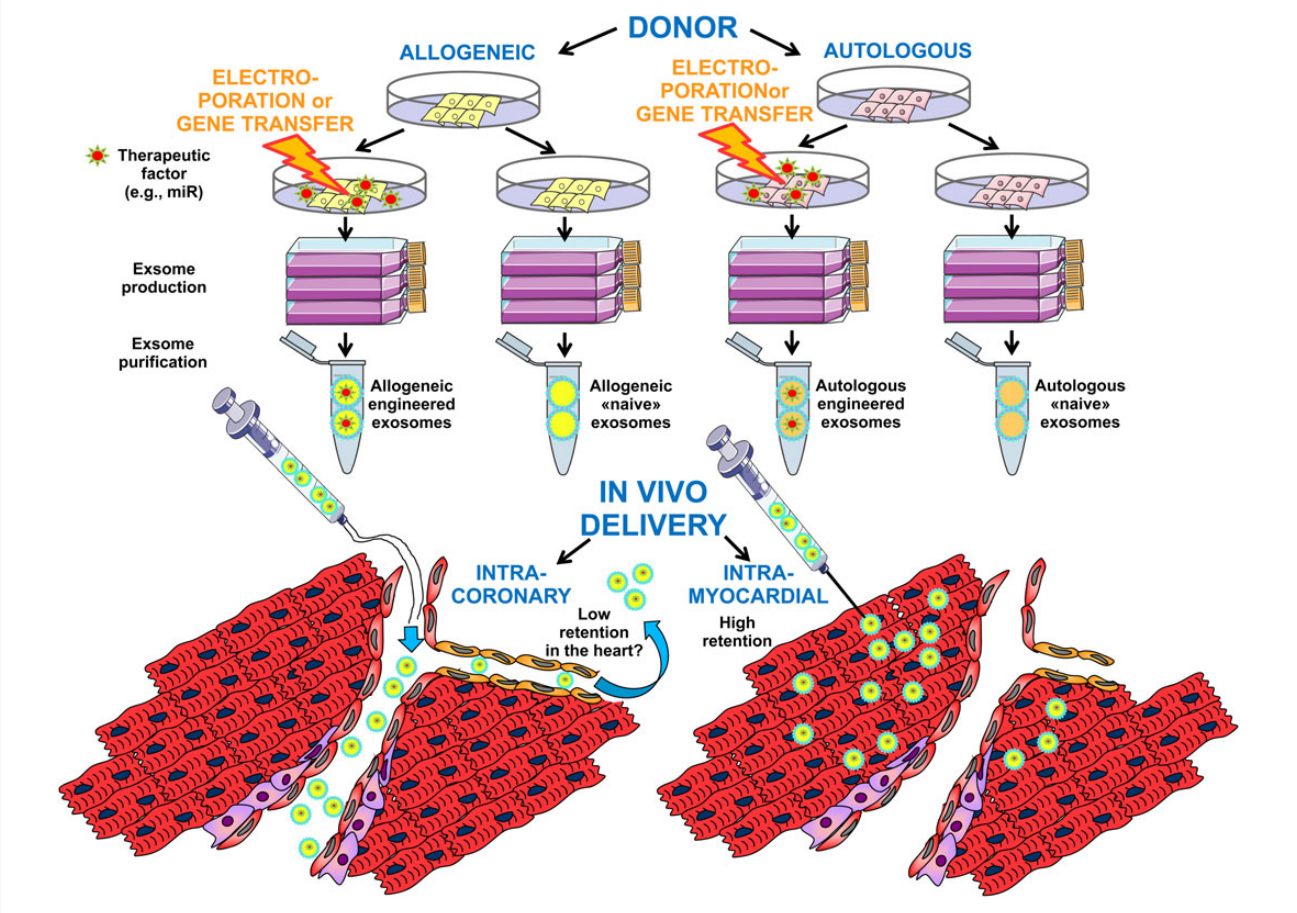

Figure 4 Exosome-based clinical applications. Exosome-producing cells are from autologous or allogeneic donors. Exosomes are released from either unmodified or engineered cells enriched in a therapeutic factor. Intramyocardial, but not intracoronary, delivery was beneficial in a mini-pig MI model.[50] Hypothetically, intracoronary exosomes may be poorly retained in the heart.

of the parent cell. Further studies addressing the respective contributions of exosomes and soluble factors in paracrine cell activities are warranted.

Future perspectives

Exosomes are of interest to cardiovascular medicine for three major reasons: in the regulation of physiological processes, as diagnostic markers, and as therapeutic candidates. Previous clinical applications included phase-I trials of anti-tumour vaccination in cancer patients using exosomes from autologous dendritic cells primed with tumour antigen *ex vivo*.[44] For future applications, it will be essential to define whether exosomes are considered the active drug components or serve as drug delivery vehicles. Taking the latter approach, exosome-producing cells can be engineered using electroporation or genetic modifications to release exosomes enriched in therapeutic nucleic acids and proteins, respectively. Artificial exosome mimetics manufactured using clinical-grade synthetic lipids and recombinant proteins to produce pharmaceutically acceptable drug delivery vehicles are under investigation.[45]

Additional issues include exosome manufacturing procedures, allogeneic cellular sources, and delivery techniques (*Figure 4*). Exosomes used in clinical trials of cancer were produced using Good Manufacturing Practice-compatible protocols. Ultrafiltration and size-exclusion methods are promising for large-scale exosome preparations.[46,47]

In patients after acute MI, cardioprotective exosomes would be best administered in an emergency setting. This would only be feasible using 'off-the-shelf' allogeneic exosomes (unless autologous exosomes were banked in advance). Although exosomes express histocompatibility antigens, they appear to be hypo-immunogenic. We recently compared the immunogenicity of EVs from xenogeneic (human) or allogeneic (rat) CDCs.[48] Repeated subcutaneous injections of EVs from the human cells induced progressive humoral and cell-mediated immune responses, as expected, although at lower levels compared with injections of the parent cells. Allogeneic EVs did not induce significant immune responses after repeated dosing. Moreover, the open-label phase-I portion of the ALLSTAR trial showed that intracoronary infusion of allogeneic CDCs is safe, with minimal or no measurable immune reactions.[49] These findings suggest that exosomes from allogeneic CDCs could be safely used in clinical applications.

Another issue is the delivery technique. We recently reported that intramyocardial injection of CDC-exosomes 30 min after coronary occlusion and reperfusion significantly reduced infarct size in mini-pigs, whereas intracoronary exosomes did not.[50] The reason for this is unclear. Hypothetically, intracoronary exosomes pass through the coronary circulation but are retained poorly in the heart. Unpublished data from our laboratories using isolated-perfused rat hearts show that CPC-exosomes added to the perfusate are taken up by cardiomyocytes. However, these data in isolated-perfused hearts cannot be compared directly with *in vivo* data. Thus, poor efficacy of intracoronary delivery may be a major limitation of exosomes for clinical applications, until and unless some of the obstacles are overcome.

In conclusion, exosomes isolated from CPCs and other progenitor cells hold tremendous promise for cardioprotection. Exosome-based approaches could 'take cells out of cell therapy'. Intramyocardial injection is the most suitable route of exosome administration to the heart, whereas intracoronary delivery is inefficacious using current techniques.

Authors' contributions

L.B., E.M., G.V. acquired the data. L.B., E.M., G.V. conceived and designed the research. G.V. drafted the manuscript. L.B., T.M., E.M. made critical revision of the manuscript for key intellectual content.

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