BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-21-161

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Article Type: Mini Review

Keywords: biomimetics; extracellular vesicles; exosome; cardiovascular disease; regenerative medicine

Corresponding Author: Kiwhan Kwon

Authors: In Sook Kang[1], Kiwhan Kwon[1,*]

Institution: [1]Department of Internal Medicine, School of Medicine, Ewha Womans University, Seoul 07804, Republic of Korea,

Potential application of biomimetic exosomes in cardiovascular disease; focused on ischemic heart disease

In Sook Kang and Kihwan Kwon*

Department of Internal Medicine, School of Medicine, Ewha Womans University, Seoul 07804, Republic of Korea.

Running Title: exosomes in cardiovascular disease

Address for Correspondence: Kihwan Kwon

Department of Internal Medicine, Mokdong hospital, School of Medicine, Ewha Womans University, Seoul 07804, Republic of Korea.

Telephone: (82-2)-2650-2640; FAX: (82-2)-2650-5424; E-mail: kankadin@ewha.ac.kr

Abstract:

Cardiovascular disease, especially ischemic heart disease, is a major cause of mortality worldwide. Cardiac repair is one of the most promising strategies to address advanced cardiovascular diseases. Despite moderate improvement in heart function via stem cell therapy, there is no evidence of significant improvement in mortality and morbidity beyond standard therapy. The most salutary effect of stem cell therapy are attributed to the paracrine effects and the stem cell-derived exosomes are known as a major contributor. Hence, exosomes are emerging as a promising therapeutic agent and potent biomarkers of cardiovascular disease. Furthermore, they play a role as cellular cargo and facilitate intercellular communication. However, the clinical use of exosomes is hindered by the absence of a standard operating procedures for exosome isolation and characterization, problems related to yield, and heterogeneity. In addition, the successful clinical application of exosomes requires strategies to optimize cargo, improve targeted delivery, and reduce the elimination of exosomes. In this review, we discuss the basic concept of exosomes and stem cell-derived exosomes in cardiovascular disease, and introduce current efforts to overcome the limitations and maximize the benefit of exosomes including engineered biomimetic exosomes.

Keywords: biomimetics; extracellular vesicles; exosome; cardiovascular disease; regenerative medicine

INTRODUCTION

Ischemic heart disease is a public health concern worldwide (1). The prognosis of ischemic heart disease has greatly improved with the development of drugs and early reperfusion therapy. However, despite successful revascularization and optimal medical treatment, secondary myocardial damage due to reperfusion injury, and progression to heart failure are persistent challenges (2).

Myocardial reperfusion injury is defined as myocardial damage triggered by the restoration of coronary blood flow after an ischemic episode (2). The mechanism of reperfusion injury is related to the generation of huge amounts of reactive oxygen species in the ischemic zone (3, 4), which is a major contributor to mitochondrial damage, apoptosis and death of cardiomyocytes, and exacerbation of left ventricular remodeling and heart failure (2, 5). The main therapeutic options available to patients with myocardial infarction are reduction of infarct size and myocardial injury, repair of damaged myocardium, as well as minimization of myocardial remodeling.

Several studies in the early 2000s reported the potential of cardiac regeneration previously thought impossible. They included reports of the presence of adult cardiac stem cells, development of clinical methods for the isolation and expansion of human cardiac stem cells, and evidence supporting cardiomyocyte renewal in humans based on the interpretation of the integration of carbon-14, generated by nuclear bomb tests during the Cold War (6-8).

Patients with end-stage heart disease refractory to conventional treatments have no choice but to undergo heart transplant. To improve the prognosis of these patients, stem cell therapy has been used for cardiac tissue repair with mesenchymal stem cells, cardiac progenitor cells, and chemically engineered cells (9-12). Despite the feasibility of clinical trials showing moderate improvement in cardiac parameters, a significant effect on mortality and morbidity

beyond standard therapy has yet to be demonstrated (10, 13-15).

The limitations of cell therapy are mainly attributed to the poor retention of the transplanted cells and the significant entrapment of cells in non-targeted organs, such as lung, liver, and spleen (11). Further, despite improvement in cardiac function, there is little evidence to suggest that the injected cells are engrafted and differentiated into cardiomyocytes (16). Hence, based on the current consensus, the paracrine effects of delivered exogenous cells play a major role in augmenting the regenerative potential of cardiac tissues (10, 17, 18).

Extracellular vesicles (EVs), including a variety of small, lipid bilayer membrane-enclosed vesicles originating from the parent cells, facilitate cell-to-cell communication (19, 20). The specificity of exosomes, EVs with a diameter of about 30-150 nm, depends on their origin cells; they act as cellular cargo containing proteins and genetic information (20, 21). Previous studies showed that exosomes regulate cardiac repair after myocardial infarction via local and distant micro-communication (20). Furthermore, coronary and systemic platelet-derived microparticles from patients with ST-elevation myocardial infarction correlated with thrombus score and represented potential biologic markers of ongoing thrombus (22).

Nonetheless, exosomes are associated with several limitations before they can be used as potential biomarkers and therapeutic agents in cardiovascular diseases. First, it is difficult to obtain high-purity exosomes suitable for clinical application. No acceptable standard method or specific markers are currently available for exosome isolation. Batch-to-bath variation is another challenge. Second, changes in the micro-environment may alter exosome cargo content or functional potency (23). Therefore, it is difficult to consistently obtain exosomes with the same quantity and quality. Third, it is nearly impossible to obtain a large amount of the same exosomes from limited cells. Consequently, even after obtaining good preclinical results with high-quality exosomes, it is difficult to perform preclinical and clinical studies

that require a stable and steady supply of large quantities of exosomes of desirable quality (19).

To address these issues, we reviewed the characteristics of exosomes, strategies to overcome the limitations, and the clinical application of exosomes in cardiovascular disease.

GENERAL CHARACTER OF EXOSOMES

Definition, isolation, and characterization

EVs are secreted by nearly all cells and exist in all biological fluids (24). EVs can be classified into three subpopulations: (1) exosomes measuring approximately 30 to150 nm in diameter, which are released by exocytosis of multivesicular bodies, and composed of a lipid-bilayer membrane and internal core, (2) microvesicles (around 100-1000 nm) shed from the plasma membrane, and (3) apoptotic bodies (> 1.0 μm) derived from apoptotic cells (18, 25). Exosomes carry various molecular cargo of their origin cells including proteins, lipids, mRNA, and microRNAs (miRNAs) (24, 25). The uptake of exosomes into recipient cells is mediated via direct fusion, receptor binding, lipid rafts, and cellular release (18).

Exosomes can be isolated via high-speed ultracentrifugation, precipitation, size exclusion chromatography, density gradient, and immune affinity capture. The principle of exosome isolation is mainly based on particle size and density of the exosomes (26). These methods facilitate the isolation of exosome-enriched subpopulation, but it is rarely possible to specifically isolate exosomes from other subpopulations of EVs (27). Furthermore, lipoproteins derived from serum or blood samples are similar to exosomes in size and density, which hinders isolation of pure exosomes from these samples (28). The properties of exosomes derived from the multivesicular bodies of the parent cell increase their value as

biomarkers or therapeutic agents, while the absence of exosome-specific markers may hinder the isolation of standardized high-quality exosomes.

Hence, exosome characterization is very difficult due to the heterogeneity and size variation, and the difficulty associated with cargo profiling (29). In addition to morphological results obtained via electron microscopy and size distribution by single-particle analysis, exosomes are generally characterized by the presence of tetraspanins (CD9, CD81, and CD63), heat shock proteins (HSP) including HSP 70, immune regulatory molecules (MHC class I, II), calcium-dependent annexin V and endosomal proteins such as syntenin-1 and TSG101 (19, 29, 30).

Cellular cargo and *in vivo* distribution of exosomes

Since the first reports of exosomes carrying mRNAs and miRNAs (31, 32), studies investigating the role of exosomes in genetic exchange between cells and intercellular communication have been active. In particular, the salutatory cardiac effects of stem-cell therapy are mainly attributed to paracrine effects via exosomes rather than direct cellular mechanisms (10, 23). Cell-free therapy using exosomes is comparable to stem-cell therapy in terms of regenerative potential without disadvantages such as tumorigenicity, immune rejection, and ethical issues. Furthermore, exosomes are durable and azoic entities unlike cells (29, 33).

Although the clinical and therapeutic application of exosomes is important, studies have seldom reported the accurate biodistribution and pharmacokinetics of exosomes administered *in vivo*. Systemically injected exosomes are cleared rapidly from the blood by circulating phagocytes. Exosomes display prolonged and sustained retention in tissues such as liver and spleen longer than 24 hours (34). Exosomes delivered systemically undergo rapid distribution initially, followed by a longer elimination phase via hepatic and renal routes within six hours

(35). However, several factors such as cellular origin, host condition, membrane composition of the exosome, and imaging methods may modulate the biodistribution and pharmacokinetics of exosomes administered *in vivo*, underscoring the need for careful approach and interpretation for effective therapeutic application (34, 36). In one study, intravenous injection of high-dose exosomes (> 400 μg) induced asphyxiation due to the accumulation of exosomes in the lungs in a murine model (36).

VARIOUS STEM CELL-DERIVED EXOSOMES IN CARDIOVASCULAR FIELD

Exosomes from mesenchymal stem cells

Mesenchymal stem cells (MSCs) are some of the most widely used stem cells due to the easy availability of accessible tissues such as bone marrow and fat, in addition to differentiation into various lineages (37-39). They are currently available for treatment of patients with acute myocardial infarction after successful revascularization (40).

Several preclinical studies demonstrated that the increased expression of exosomal miR-21-5p, miR-22, and miR-29 was related to improve cardiac tissue contractility, decrease ischemia-induced apoptosis, and reduce infarct size by attenuating tissue fibrosis (41-43). Exosomes derived from hypoxic human MSCs increased the level of miR-26a compared with exosomes from normoxic human MSCs, which attenuate the infarct size and reduce arrhythmia by suppressing GSK3β expression in the ischemia/reperfusion injury model (44).

Exosomes from cardiosphere-derived/cardiac progenitor cells

Cardiosphere-derived cells (CDCs), which have been isolated and expanded from human heart biopsy specimens and grow as self-adherent clusters *in vitro*, are considered cardiac stem cells exhibiting self-renewal and regenerative potential (45, 46). CDCs injected into infarcted

mouse hearts significantly improved cardiac function, cell engraftment, and myogenic differentiation rates than MSCs, and exhibited a balanced profile of paracrine factors (39).

Exosomes derived from cardiac progenitor cells (CPCs) exposed to hypoxia promote angiogenesis and upregulate the expression of miRNA clusters, which improved cardiac function and reduced fibrosis in ischemia/reperfusion injury (46, 47). Oxidative stress-treated CPCs also secrete exosomes containing cardioprotective miRNAs including miR-21 and miR-451(48).

Exosomes from induced pluripotent stem cells

Induced pluripotent stem cells (IPSCs) are reprogramed from somatic cells using four stem cell transcription factors (Oct4, Sox2, Klf4, and c-Myc) without ethical concerns, while maintaining pluripotency similar to embryonic stem cells. However, IPSCs not free from tumorigenicity (49, 50).

Exosomes derived from IPSCs deliver cardioprotective miRNAs, including miR-21 and miR-210, which suppress caspase 3/7 activation and inhibit oxidative stress-induced apoptosis of cardiomyocytes in the ischemic myocardium (51). Exosomes from IPSC-derived cardiac progenitors carry 16 highly abundant miRNAs, which are related to increasing cardiomyocyte survival, proliferation, endothelial cell migration, and improved cardiac function (52).

Exosomes from the heart and systemic cells

Exosomes are secreted by major heart cells, including cardiomyocytes, endothelial cells, fibroblasts, and smooth muscle cells. In addition, immune cells and platelets also release exosomes (19).

Hypoxic conditions induce the expression of HSP 60 in cardiomyocyte-derived exosomes and alter the exosomal composition of mRNAs and proteins of endothelial cells (53, 54). Pressure and volume overload induce cardiac hypertrophy via intercellular communication between the fibroblasts, endothelial cells, smooth muscle cells, and inflammatory cells (19). Platelet-derived exosomes regulate coagulation response and may promote atherosclerosis (55, 56). Importantly, exosomes from bone marrow-derived macrophages may promote the resolution of vascular inflammation and atherosclerosis via miRNA cargo (57).

These properties of exosomes suggest a potential biomarker and therapeutic role in atherosclerosis and cardiovascular disease.

EXOSOMES FOR CLINICAL APPLICATION IN CARDIOVASCULAR DISEASE

Exosomes as biomarkers of heart disease

Exosomes share the characteristics of their parent cells and exist in all body fluids due to their high accessibility, and thus designated 'lipid biopsy', and hold great promise and potential in applications for the management of ischemic heart disease (19, 24).

Blood-borne biomarkers such as circulating miRNA in addition to electrocardiogram facilitate the evaluation of persistent low-grade myocardial ischemia without concomitant cell death, subclinical myocardial infarction, microvascular angina, or acute coronary syndrome without ST-segment elevation (58, 59). Importantly, the expression of miR-126 and miR-199a expression in exosomes is predictive of a cardiovascular event in patients with stable angina (60), and miR-208a level correlates with acute coronary syndrome (61). Further, previous studies demonstrated the elevation of specific exosomes in atherosclerosis and thrombotic occlusion of the coronary artery (62-64).

The qualitative and quantitative changes in exosomes are important in cardiovascular disease.

The number of coronary endothelial cells or platelet-derived exosomes increases according to the degree of thrombosis and ischemic insult (22, 65). The increase in the number of exosomes derived from various cells associated with atherosclerosis has important implications for the prediction of cardiovascular mortality (66). Hence, exosomes derived from various cardiac cell types represent novel biomarkers for ischemic heart disease and atherosclerosis.

However, the challenge is that cardiovascular disease is not the only factor that is associated with changes in the quantity or quality of exosomes in the related cells. Various metabolic conditions and comorbidities, such as hypertension, diabetes, hyperlipidemia, and obesity, can also affect the properties of exosomes (67, 68). A case-control study demonstrated that patients treated with statin carried a lower number of circulatory MVs carrying activated cells than untreated patients with similar cholesterol levels (69). The result shows that exosomes can be meaningful as a biomarker in metabolic conditions and play a significant role in the evaluation of therapeutic effects. However, they also show the difficulties associated with the interpretation of multifactorial diseases.

Exosomes are potential biomarkers in clinical practice due to the various advantages described above. Nonetheless, the quantity and quality of exosomes *in vitro* can vary depending on factors related to parent cells and the culture environment, whereas *in vivo*, they are affected by patient's condition, comorbidities, and acute or chronic disease. In addition, specific exosome subpopulations in various diseases and environments should be defined, and reproducibility in various laboratories must be demonstrated. Furthermore, scalability and reproducibility are critical since the low yield of exosomes hampers active clinical investigations (19). As a result, successful clinical transition from laboratory test is difficult and only two clinical trials have been found to date; one is ‘differential expression and analysis of peripheral plasma exosome miRNA in patients with myocardial infarction’ by using exosomes in peripheral blood of patients (NCT04127591, not yet recruiting) and the other is

‘role of exosomes derived from epicardial fat in atrial fibrillation by using patients’ epicardial fat tissue (NCT03478410).

Therapeutic potential of exosomes in ischemic heart disease

Regenerative therapies and drug delivery using exosomes in various heart diseases such as advanced heart failure and ischemic heart disease have been attempted; however, the results are still in their infancy. However, clinical trials in oncology outnumber those in cardiovascular diseases. The feasibility and safety of large-scale production of autologous dendritic cell-derived exosomes has been demonstrated in a phase 1 clinical trial involving patients with metastatic melanoma (70). The proposed role of exosomes in the field of cardiovascular disease is summarized in Table.

Clinical studies in myocardial infarction were conducted using MSCs and CPCs as described above, with reduction of scar tissue and improvement in cardiac function; however, no improvement in mortality was evident compared with standard treatment (13, 14, 40). Several preclinical studies have reported that intramyocardial injection of exosomes improved cardiac function or alleviated the severity of myocardial damage in the myocardial infarction model (20, 39). In dilated cardiomyopathy, the cardiac function was improved by the intravenous administration of exosomes of cardiosphere-derived stem cells (71).

Computational modeling approaches have been used recently to elucidate the complexity and to predict functional responses of exosomes for preclinical/clinical use (72, 73).

Limitations of exosomes for therapeutic/clinical use

There are several limitations in using exosomes as biomarkers or therapeutic agents in cardiovascular disease. (1) The absence of standard operating procedures for exosome isolation, storage, characterization, and analysis is a concern (29). (2) In the absence of a gold

standard to distinguish the specific subpopulations, it is hard to specify the origin of exosome subpopulations including cardiomyocytes, endothelial cells, smooth muscle cells, or immune cells. (3) It is difficult to obtain a large yield of pure exosomes containing identical quality and quantity of cargo from the limited population of cells. Microenvironmental changes such as stress and culture conditions influence the quantity and function of the exosomes. (4) A majority of exosomes delivered systemically are eliminated via liver, lung, and spleen, before reaching the target. (5) The pharmacokinetics of exosomes is unclear (34), and it is difficult to predict and control intercellular communication to optimize the cardioprotective effect via systemic administration.

Studies are ongoing to overcome the limitations of exosomes and to enable practical applications for clinical use via modification or fabrication of biomimetic exosomes.

ENGINEERED EXOSOMES

Currently, several strategies to genetically engineer and optimize cargo, improve targeted delivery, and reduce the elimination of exosomes are underway. A summary of current strategies and techniques in biomimetic exosome engineering are presented in Figure and discussed below.

Exosome engineering by the modification of parental cells

1. Genetic manipulation

Genome modification via transfection of specific mRNAs, miRNAs, or proteins within the parental cells enables the production of specific cargo-rich exosomes (18, 74). Loading miR-126-3p lentiviral vector in the MSCs resulted in the secretion of potent MSC-derived exosomes

than control *in vitro* angiogenesis and tube formation (75). Genetic manipulation can enhance not only the functional aspects of the exosome but also improve the yield. The amount of exosome released from donor cells can be controlled by regulating the expression of Rab protein, which is related to the exosome secretion pathway (76).

2. Exogenous and environmental stimulation

The therapeutic potency of exosomes can be enhanced by adding synthetic bioactive molecules such as growth factors, cytokines, and drugs into the cell culture medium. MSCs cultured with erythropoietin increased exosome yield by 33% compared with the control, and also enriched the desirable miRNAs including miR-299, miR-499, miR-302, and miR-200 (77).

Another strategy is to improve scalability and therapeutic potential via external stimulation using three-dimensional culture, hypoxic stimulus, and bioreactor systems (46, 74, 78).

Modification of isolated exosomes

1. Modification of exosome cargo

Two major methods include passive and active cargo loading. Passive cargo loading is defined as the co-incubation of the desired compounds with exosomes. The compounds can be loaded via diffusion and hydrophobic interaction between the lipid membranes of exosomes and molecules (e.g., hydrophobically modified small interfering RNAs), (79). Active cargo loading entails temporary disruption of the exosome membrane to facilitate the diffusion of the desired compounds via electroporation, sonication, extrusion, freeze-thaw cycles, and saponin-assisted loading (74, 79). Active cargo loading is more effective than passive loading in terms of loading capacity; however, possible membrane damage is a limitation (79).

2. Modification of exosome surface

The surface structures of exosomes play a critical role in their biodistribution, intercellular communication, and cell targeting (18, 74). Functionalized exosomes with increased therapeutic utility can be produced via modification of exosome surface.

Gene cloning can be used to insert a homing peptide sequence into a gene. The homing peptide can also be anchored on an exosomal surface (18). Anchoring homing peptides to modified exosome membranes improves targeted delivery. Homing peptides vary depending on the target cells, and their physiologic and pathologic conditions (80-82). These peptides can be used to induce changes in surface composition to improve targeted delivery, modification of cargo such as proteins or lipids in exosomes, or anchor an imaging agent for *in vivo* tracking (18, 83).

Surface peptide engineering using cardiac homing peptides can improve targeting to ischemic myocardium (18, 80). Vandergriff et al. successfully targeted regenerating exosomes to infarcted myocardium by conjugating the exosomes with cardiac homing peptides via dioleoylphosphatidylethanolamine-N-hydroxysuccinimide (DOPE-NHS), resulting in improved outcomes with reduced fibrosis, increased cellular proliferation, and angiogenesis (80). In addition to homing peptides, molecular platforms that directly coat antibodies or other biological ligands on the surface of exosomes also enhance homing to ischemic myocardium (84).

Alleviation of exosome clearance

Nearly 95-99% of systemically delivered exosomes are eliminated before target delivery, and the mononuclear phagocyte system (MPS) plays a major role in this elimination (18, 85). Therefore, most efforts to avoid clearance by tailoring biomimetic exosomes include modification of size or polymer to avoid phagocytosis and deactivate the MPS (18).

The optimal size of nanoparticle for cellular entry is approximately 50 to100 nm (86). Polyethylene glycol (PEG) including the first FDA-approved liposome-based nano-drug is the most common polymer-modifier used (87). Engineering of hybrid PEGylated exosomes via membrane fusion with liposomes increased vesicle half-life and decreased vesicle immunogenicity (88). However, PEG-related hypersensitivity and paradoxical accelerated blood clearance are a concern (89).

Pretreatment or combined use of MPS-modulating agents, such as chloroquine, with nanoparticles led to macrophage preconditioning and improved nano-delivery (90). Another strategy for deactivating MPS includes blocking clathrin-dependent endocytosis by the liver and spleen, which is known to play a major role in exosome clearance. Wan et al. demonstrated exosome loading with siRNA against clathrin heavy chain 1 to improve their accumulation in the myocardium (91). Mimicking ‘self’ with CD47-derived peptide or natural cell-derived membrane coating also prevents phagocytosis (92).

Fabrication of artificial exosomes

Synthetic nanoparticles, such as liposomes loaded with erythropoietin and CD15s and ligustrazine ethosome patches, have shown salutary effects in models of myocardial infarction by improving cardiac function and reducing infarct size and arrhythmia (93, 94).

Fabrication of hybrid exosomes, also called ‘fusogenic liposomes' is another strategy to attenuate myocardial ischemia/reperfusion injury. Platelet-inspired nanocells with membrane-modified prostaglandin E2 and carrying cardiac stromal cell-secreted factors as cargo showed improved homing to injured myocardium and augmented cardiac function after intravenous injection (95). Tang et al. fabricated artificial nanoparticles from polylactic-co-glycolic acid, (PLGA) and conditioned media from human cardiac stem cells as ‘core shell’ design and

cloaking the core shells with cardiac stem cell membrane fragments to avoid immune reaction to mimic cardiac stem cells (96). As a result, they showed that artificial nanoparticles simulate paracrine effects and preserve viable myocardium and augment cardiac functions.

Application of functionalized biomaterial platforms

Functionalized biomaterial platforms have been proposed to minimize the clearance and increase the half-life of exosomes for improved target delivery with sustained local release. One study reported that an engineered hydrogel patch capable of sustained release of exosomes from IPSC-derived cardiomyocytes reduced arrhythmic burden, promoted ejection fraction recovery, and decreased cardiomyocyte apoptosis (97).

CONCLUSION

Exosomes are promising therapeutic agents used in myocardial repair after ischemic/reperfusion injury, based on their paracrine effects while maintaining the character of the parent cells. Application of exosomes to improve cardiac function in patients with advanced heart failure is feasible in the near future. Exosomes are also promising and potent biomarkers since they exist in almost all biological fluids in varying quantities and the quality of the cargo depends on the metabolic conditions of the host.

However, the clinical application of exosomes is hampered by their heterogeneity, low yield, and difficulties associated with target delivery. Effective exosome-based therapeutic application requires elucidation of the exosome pharmacokinetics in the human body.

It is expected that these limitations can be overcome via biomimetic exosome manipulation, which is being actively studied currently in cardiovascular diseases. Further, these approaches are expected to herald a new era of personalized therapy using custom-made exosomes.

ACKNOWLEDGMENTS

None

CONFLICT OF INTEREST

The authors have no conflicting interests.

Table. Future perspective for cardiac therapeutics of engineered exosomes

More predictable pharmacokinetics and biodistributions
Overcoming heterogeneity of natural exosome isolation and purification
Cargo optimization, improve targeted delivery, and reduced elimination
Clinical translation through exosome fabrication with high reproducibility and scalability
Personalized therapy through custom-made exosomes

Figure legend

Figure . Overview of biomimetic exosome engineering techniques. Numerous techniques have been attempted to enhance the therapeutic efficacy and scalability of exosomes. In this figure, we simplified four major concepts: modified parental cells, modified exosomes, avoidance of MPS (mononuclear phagocyte system), and artificial exosomes. PEG; polyethylene glycol

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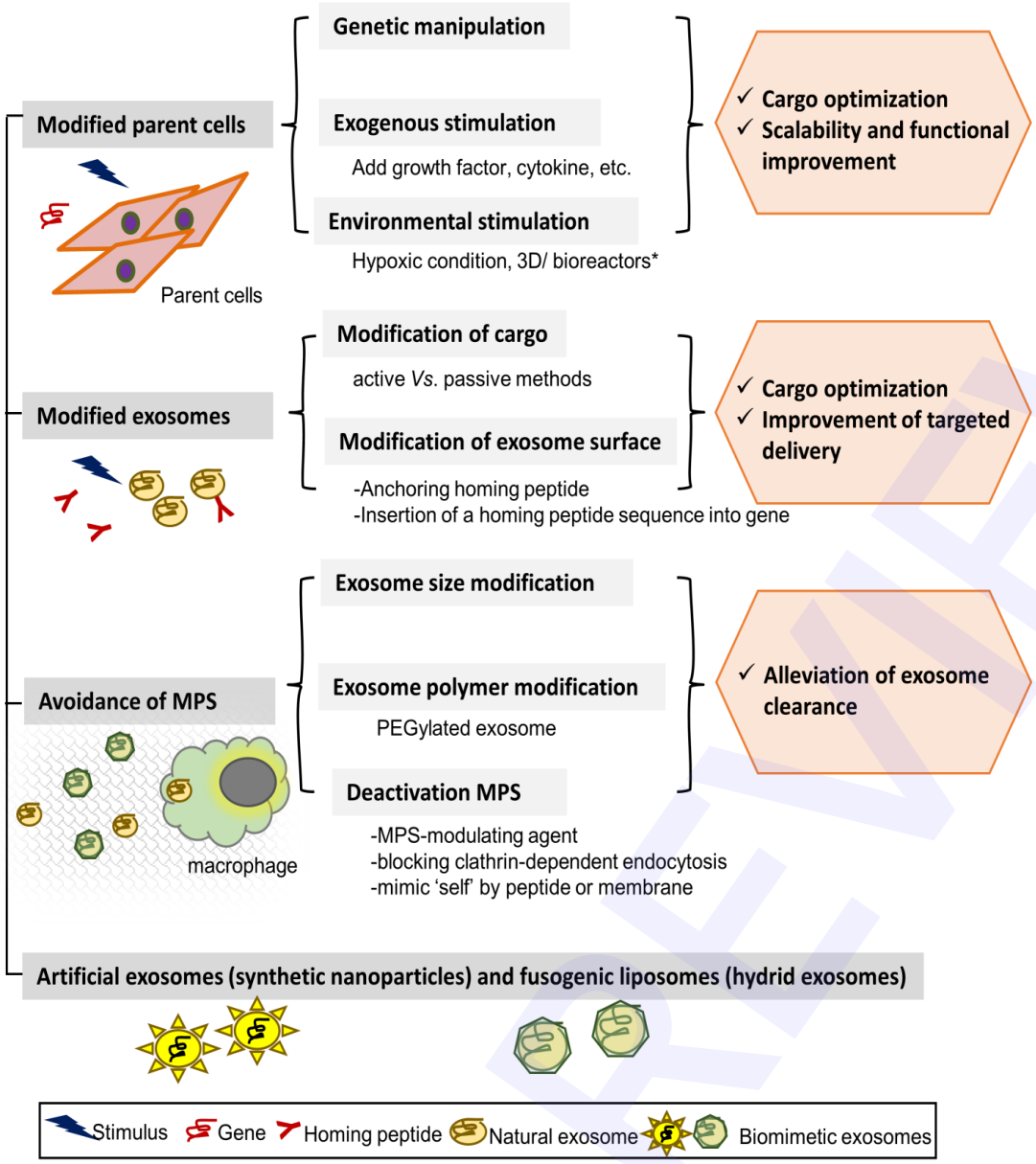

Fig. 1.