JACC: BASIC TO TRANSLATIONAL SCIENCE
VOL. 2, NO. 6, 2017
ISSN 2452-302X
https://doi.org/10.1016/j.jacbts.2017.08.004

STATE-OF-THE-ART REVIEW

Endothelial- and Immune Cell-Derived Extracellular Vesicles in the Regulation of Cardiovascular Health and Disease

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SUMMARY

Intercellular signaling by extracellular vesicles (EVs) is a route of cell-cell crosstalk that allows cells to deliver biological messages to specific recipient cells. EVs convey these messages through their distinct cargoes consisting of cytokines, proteins, nucleic acids, and lipids, which they transport from the donor cell to the recipient cell. In cardiovascular disease (CVD), endothelial- and immune cell-derived EVs are emerging as key players in different stages of disease development. EVs can contribute to atherosclerosis development and progression by promoting endothelial dysfunction, intravascular calcification, unstable plaque progression, and thrombus formation after rupture. In contrast, an increasing body of evidence highlights the beneficial effects of certain EVs on vascular function and endothelial regeneration. However, the effects of EVs in CVD are extremely complex and depend on the cellular origin, the functional state of the releasing cells, the biological content, and the diverse recipient cells. This paper summarizes recent progress in our understanding of EV signaling in cardiovascular health and disease and its emerging potential as a therapeutic agent. (J Am Coll Cardiol Basic Trans Science 2017;2:790–807) © 2017 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Cardiovascular disease (CVD) still represents the leading cause of mortality worldwide. The underlying disease, atherosclerosis, is initiated and propagated by continuous damage of the vascular endothelium, leading to endothelial activation and apoptosis, the development of endothelial dysfunction, and subsequent atherosclerotic lesion formation (1). Endothelial cell (EC) injury is a key element in the complex pathophysiology of atherogenesis and triggers the release of EC-derived extracellular vesicles (EVs) such as exosomes and microvesicles (MVs) (2). Accordingly, patients with vascular diseases associated with systemic endothelial damage, such as atherosclerosis, show significantly increased levels of circulating EVs (3,4). However, EVs are not simply inactive debris that reflect cellular activation or injury. EVs can transfer proteins, cytokines, mRNA, or noncoding RNA such as microRNA (miRNA) or long noncoding RNA to target cells and influence their function and phenotype (5,6). Accordingly, the role of EVs has changed from being only a marker of vascular integrity toward being relevant effectors in intercellular vascular signaling (7,8). In CVD, EVs have been shown to

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All authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the *JACC: Basic to Translational Science* author instructions page.

Manuscript received July 7, 2017; revised manuscript received August 14, 2017, accepted August 14, 2017.

contribute to disease development and progression by promoting initial lesion formation, intravascular calcifications, plaque progression, and thrombus formation after rupture. In contrast, numerous studies have demonstrated that certain subtypes of EVs can mediate vascular protection and endothelial regeneration (9). In line with these findings, EVs released by progenitor or mesenchymal stem cells have been shown to improve cardiac function after myocardial infarction in experimental studies, highlighting the therapeutic potential of EVs in cardiovascular pathologies (10). This review summarizes current knowledge of EVs as regulators of cardiovascular health and disease and potential opportunities for therapeutic use.

BIOGENESIS OF EXTRACELLULAR VESICLES AND THEIR INTERACTIONS WITH TARGET CELLS

EVs are membrane vesicles secreted from cells that contain intracellular contents (11). Cells can release a broad range of vesicles with diverse features. This review focuses on 2 major types: MVs and exosomes. MVs are large (>150 nm) vesicles that are released by budding from the plasma membrane, whereas exosomes are smaller (30 to 100 nm) and originate from the endosome (12). However, there is no strict cutoff value that distinguishes MVs from exosomes by vesicle size, which can differ in diverse studies (12).

Exosomes represent a homogeneous population of vesicles that are formed by inward budding of the multivesicular body (MVB) membrane. Exosome biogenesis is mediated mainly by the endosomal sorting complex required for transport (ESCRT) protein (13) or lipid ceramide and neutral sphingomyelinase, the enzyme that converts sphingomyelin to ceramide (14). Cargo sorting into exosomes involves ESCRT and associated proteins such as tumor susceptibility gene 101 protein (TSG101) and ALG-2-interacting protein X (ALIX) and small GTPases such as Rab7a and Rab27b (15–17). Exosomes are liberated into the extracellular space following fusion of MVBs with the cell membrane, regulated by Rab27A, Rab11, and Rab31 (18,19). MVs represent a relatively heterogeneous population of vesicles formed by outward budding of the cell membrane. This process is regulated by membrane lipid microdomains and regulatory proteins such as ADP-ribosylation factor 6 (ARF6) (20). EVs can be regarded as intercellular messengers for various biological processes. Several routes of interaction between EVs and recipient cells have been described. First, EVs can directly activate target cell surface receptors by bioactive ligands and proteins (21–23). Second, EVs are able to transfer their biological content by membrane fusion with the recipient cell. The fusion process is regulated by the lipid composition of EV membrane, and several reports indicate that the presence of phosphatidylserine contributes to membrane fusion (24). Third, incorporation of EVs into target cells is mediated by endocytosis, pinocytosis, or phagocytosis (25). Through these interaction routes, EVs transfer their biological contents containing nucleic acids such as mRNA (26), noncoding RNAs (miRNAs [27], long noncoding RNAs [6]), proteins (28), cytokines (29), or bioactive lipids (30) (**Figure 1**).

ABBREVIATIONS AND ACRONYMS

CVD = cardiovascular disease

EC = endothelial cell

EMV = endothelial cell-derived microvesicles

ESCRT = endosomal sorting complex required for transport

IL = interleukin

miRNA = microRNA

MV = microvesicles

NO = nitric oxide

PEG = polyethylene glycol

TGF = transforming growth factor

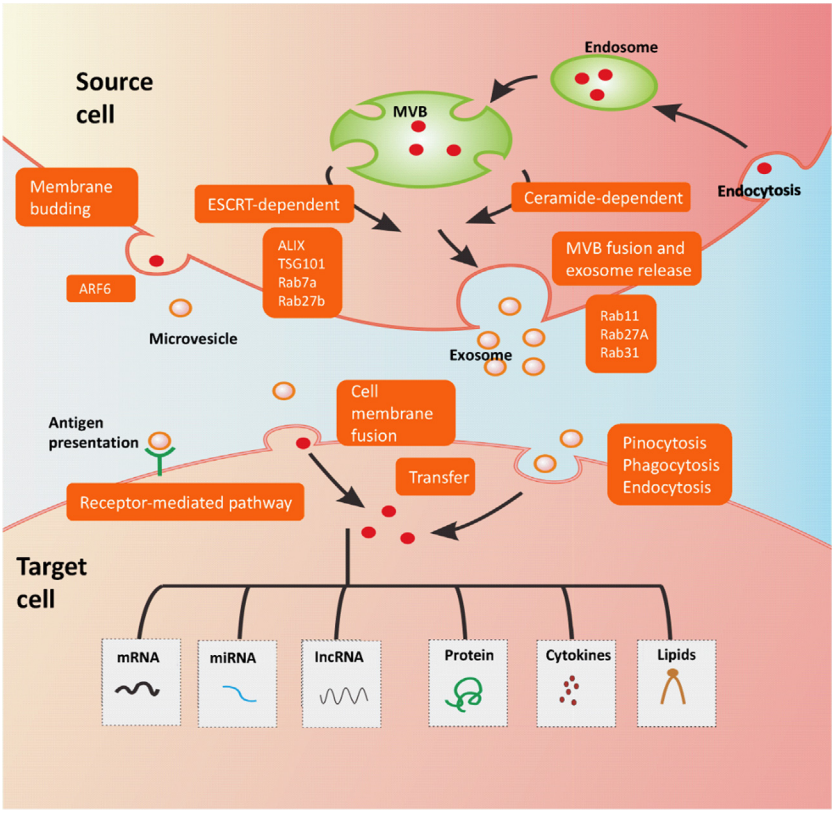
EXTRACELLULAR VESICLES AS EFFECTORS OF CARDIOVASCULAR DISEASES

In cardiovascular biology, EVs have various physiological functions, including activation of platelets and ECs, as well as regulation of inflammation and coagulation (31–34). Therefore, EVs are emerging as key players in different stages of CVD development (31,32,35). The effects of EVs in CVD are extremely complex and depend on the cellular origin, the functional state of the releasing cells, the intravesicular content, and the recipient cells (36,37). The following sections summarize the current knowledge about EVs as effectors of CV disease progression or vascular repair.

DETRIMENTAL EFFECTS OF EXTRACELLULAR VESICLES ON VASCULAR FUNCTION. Endothelial dysfunction occurs as a response to cardiovascular risk factors and represents the initial step in atherosclerosis development, the underlying pathology of CVD (38,39).

Endothelial dysfunction. Endothelial MVs have been shown to impair vasorelaxation by inhibiting nitric oxide (NO) production in target ECs. This phenomenon is mediated through a decrease in endothelial NO synthase phosphorylation and activity (40), local oxidative stress (41), or an increased NADPH oxidase activity with MVs (33) and results in impaired vascular relaxation capacities. MVs in the aforementioned studies are obtained from ECs under physiological (40,41) or pathological (33) conditions, but they all have detrimental effects on vasorelaxation. Regarding the relation between molecular contents and function of EVs, further investigation should be conducted to clarify and compare the cargoes of EVs derived under different conditions (e.g., by RNA sequencing or proteomic analysis).

FIGURE 1 EV Biogenesis and Interaction With Recipient Cells



Exosome formation starts with endocytosis, a process in which the cell membrane is pinched inward and captures bioactive molecules, resulting in the formation of the endosome. These molecules are sorted into smaller vesicles that bud from the perimeter membrane into the endosome lumen, forming vesicles; this leads to the multivesicular appearance of late endosomes and so they are also known as MVBs. From MSBs, exosome formation occurs by an ESCRT- and ceramide-dependent pathway. Cargo sorting into exosomes involves ESCRT and TSG101, ALIX, and Rab7a, and Rab27b. Exosomes are released into the extracellular space following the fusion of MVBs with the cell membrane, which is regulated by Rab27A, Rab11, and Rab31. Microvesicles are formed by the outward budding of the cell membrane, a process that is regulated by ARF6. Several routes of interaction between EVs and recipient cells have been described. First, EVs can directly activate target cell surface receptors. Second, EVs are able to transfer their biological content by membrane fusion with the recipient cell. Third, incorporation of EVs into target cells is mediated by endocytosis, pinocytosis, or phagocytosis. Using these interaction routes, EVs transfer their biological content containing nucleic acids such as mRNA, noncoding RNAs (microRNAs, long noncoding RNAs), proteins, cytokines, or bioactive lipids. mRNA = messenger RNA; MVB = multivesicular bodies.

In line with the latter findings, MVs isolated from patients with vascular (acute coronary syndrome [42]) or predisposing disease (chronic renal failure or metabolic syndrome [43,44]) were shown to induce endothelial dysfunction ex vivo in rat aortic rings. In contrast, MVs from healthy subjects did not affect endothelial function (42-44), indicating that the pathophysiological state of the releasing cell determines not only the number of released MVs but also their content and biological function. Which cell-specific MVs mainly influence endothelial function and whether isolated MVs from different cell types may have diverse biological functions need to be addressed in future studies.

Of interest, storage of human blood under standard blood-banking conditions results in accumulation of MV-encapsulated hemoglobin. These erythrocyte-derived MVs react with and degrade NO, inducing endothelial dysfunction (45). Finally, platelet-derived exosomes derived under septic conditions have been shown to mediate septic endothelial dysfunction by inducing endothelial apoptosis involving superoxide, NO, and peroxynitrite production (46). Studying EVs derived from patients

represents an important translational approach to exploring the relationship between EVs and certain diseases. However, comorbidities as well as patients' age and sex should be taken into consideration as possible confounders. Therefore, inclusion and exclusion criteria should be thoughtfully determined, and control groups must be carefully selected.

Endothelial activation, monocyte adhesion/infiltration, and inflammation. Inflammation plays a pivotal role in CVD (38,47). EVs from various cellular sources contribute to vascular inflammatory processes including endothelial activation, monocyte adhesion, and transmigration (48–51).

In vitro studies have demonstrated that MVs can induce release of the proinflammatory cytokines interleukin (IL)-6 and IL-8 from ECs and leukocytes (52,53); and promote expression of adhesion proteins ICAM-1, VCAM-1, and E-selectin, facilitating increased adhesion of monocytes (54,55) and subsequent transmigration, leading to vascular inflammation and plaque development. Mechanistically, increased adhesion of monocytes to ECs can be mediated by the MV-mediated transfer of proinflammatory molecules such as oxidized phospholipids (56), caspase-3 (57), or RANTES (regulated on activation, normal T cell expressed and secreted) protein, which is transferred from platelet MVs to endothelial target cells (58). Platelet MVs from apoptotic platelets also facilitate differentiation between resident macrophages and professional phagocytes (59). Vascular inflammatory processes involve different cells (e.g., ECs, monocytes, and platelets [48–51]). The aforementioned in vitro studies demonstrated that EVs from diverse parent cells can act on various types of target cells. In summary, vascular inflammation seems to be regulated by complex intercellular communication routes involving a network of cells and EVs. To gain more insight into these multifaceted mechanisms, coincubation of different EVs with diverse cell types may be helpful to study this "intercellular communication network." However, there is a lack of adequate in vitro models, which should be addressed in further studies.

Pathological conditions modify EV content and biological functions (60). MVs isolated from atherosclerotic plaques transfer ICAM-1 to ECs and recruit inflammatory cells, suggesting that human plaque MVs promote atherogenesis (24).

Moreover, oxidatively modified, but not native, EC-derived EVs contain proinflammatory oxidized phospholipids that elicit specific responses in ECs, leading to the adhesion of monocytes (56). In line with these findings, EC-derived EVs generated from glucose-treated cells, but not from healthy ECs, facilitated up-regulation of ICAM-1 and VCAM-1 in endothelial target cells by activating p38 in an reactive oxygen species-dependent manner (33). In light of these proinflammatory effects and the increased levels of endothelial MVs in diabetic patients, one may speculate that MVs released under pathological high-glucose conditions might represent a paracrine mediator transporting proinflammatory messages to target cells and thereby foster vascular inflammation (61,62). However, while studying the functional effects of EVs derived under pathological conditions, one must consider that the quantity and/or content of EVs may vary with different modes and durations of stimulation. Therefore, clearly defined pathological conditions (including standardized concentrations and duration of cell stimulations) as well as a careful selection of an adequate control group are mandatory to elaborate EV functions depending on the parent cell conditions.

Compared with the role of MVs, the role of exosomes in vascular inflammatory processes has been less explored (63,64). However, monocyte-derived exosomes seem to induce vascular inflammation and cell death by transferring inflammatory miRNAs into ECs resulting in a significant up-regulation of ICAM-1, CCL2, and IL-6 levels (65) and provocation of endothelial apoptosis by tissue factor release (66).

Atherosclerotic plaque development, progression, and rupture. Atherosclerotic plaque rupture with subsequent coronary thrombosis represents the ultimate step in atherosclerotic lesion progression, leading to acute myocardial ischemia. Atherosclerotic plaques can release large amounts of EVs, contributing to plaque progression and instability through various mechanisms. Plaque EVs originate mainly from leucocytes, reflecting the local inflammatory environment (67). Plaque EVs express surface antigens consistent with their leukocyte origin, including major histocompatibility complex classes I and II, and dose-dependently induce T-cell proliferation (68). Antigen-specific activation of $CD4^+$ T cells was also induced by dendritic exosomes, implicating their potential involvement in vascular inflammation and plaque development (69). Furthermore, plaque MVs carry catalytically active tumor necrosis factor (TNF)-α-converting enzyme (TACE/ADAM17) and significantly enhance the processing of its substrates TNF-α and TNF receptor, thereby promoting an inflammatory response (70). Importantly, plaque EVs from patients or in vivo models are not single-component molecules, although mainly of leucocyte origin (67), and their constitutions may also depend on the stage of plaque progression (stable or unstable?).

Therefore, standard procedures to derive and analyze plaque EVs should be established.

Local inflammation is enhanced by monocytic MVs fostering leucocyte adhesion to postcapillary venules and T-cell infiltration in atherosclerotic plaques in vivo (71,72). Exosomes from T cells also can contribute to atherosclerotic plaque development by inducing cholesterol accumulation in human monocytes by the phosphatidylserine-receptor (73).

Vascular smooth muscle cell (VSMC) proliferation plays an important role in atherosclerotic plaque development (39,74). The effect of MVs on VSMC proliferation depends on the cellular origin. In vitro-generated platelet-derived MVs promoted VSMC proliferation in a platelet-derived growth factor (PDGF)-independent mechanism with minor effects on migratory capacity (75,76). In turn, monocyte-derived MVs were shown to deliver a lethal message by encapsulated caspase-1–inducing VSMC cell death (77). More recently, calcification-competent EVs derived from smooth muscle cells, valvular interstitial cells, and macrophages have been described as mediators of vascular calcification that modulate heart valve disease and atherogenesis (78–80). Although EVs show protective effects against heart valve calcification, the potential underlying mechanisms are unknown and should be addressed in future studies.

Angiogenesis is a fundamental process in CVD, contributing to plaque instability by promoting neovascularization. Unstable plaques are characterized by an increased number of vasa vasorum mediating intraplaque hemorrhage (81). MVs isolated from human atherosclerotic plaques were shown to stimulate EC proliferation in vitro after CD40 ligation and to enhance in vivo angiogenesis. Interestingly, the proliferative effect of MVs isolated from atherosclerotic plaques was more pronounced using MVs from symptomatic patients than from patients without symptoms. Therefore, MVs could represent a major determinant of plaque vulnerability (82). Unstable human plaques contain large numbers of procoagulant MVs, originating mostly from leucocytes, erythrocytes, and VSMCs localized within the necrotic core (83). Once plaque rupture occurs, these MVs can initiate the coagulation cascade through different mechanisms (67,84): first, by the expression of tissue factor (mainly on monocytic MVs), one major initiator of blood coagulation (34,85,86); and second, by exposure to phosphatidylserine on their outward membrane layer (87). Procoagulatory effects of MVs have been demonstrated in vitro and in vivo, where they facilitated thrombus formation (88,89). The deleterious effects of EVs inducing vascular inflammation, plaque progression, and rupture are illustrated in **Figure 1**.

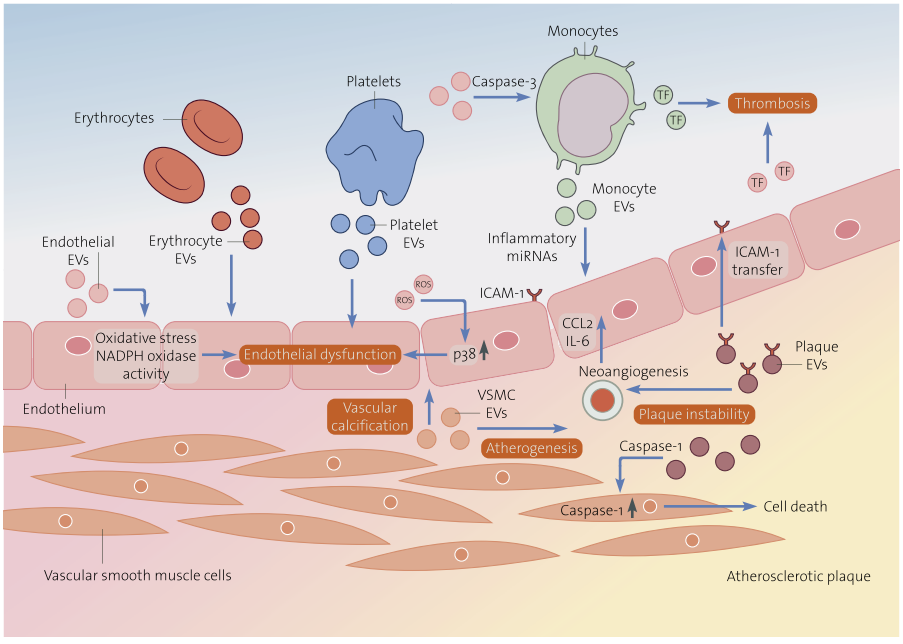
In summary, a broad body of evidence indicates the active involvement of EVs in plaque development, progression, and thrombus formation after rupture. However, there is an urgent need for additional mechanistic studies to explore how their atheroprone effects can be targeted to decelerate atherosclerotic lesion formation and rupture.

FAVORABLE EFFECTS OF EXTRACELLULAR VESICLES ON VASCULAR FUNCTION. Despite the various deleterious effects of EVs in the pathogenesis of CVD, an increasing body of evidence highlights the beneficial effects of certain EVs on vascular function. The following sections summarize the impact of EV on endothelial repair, their inhibitory effect on vascular inflammation, and their role in plaque stabilization.

Endothelial protection and vascular repair. Given that EC injury is not only a key element in the complex pathophysiology of atherogenesis but also in in-stent restenosis occurring after treatment of coronary stenosis (90,91), a mechanistic understanding of EC repair is pivotally important to develop therapeutic strategies to preserve endothelial integrity and vascular health. Several studies have shown that EVs particularly of endothelial origin can act as intercellular messenger to promote endothelial regeneration and vascular protection in vitro and in vivo.

A potential contribution of endothelial MVs in EC survival was shown by Abid Hussein et al. (92), who demonstrated that endothelial MV release is cell protective by exporting caspase-3 into MVs and thereby diminishing intracellular levels of proapoptotic caspase-3. Statins seem to facilitate endothelial health by promoting endothelial MV release in vitro (93). Nevertheless, the role of statins in endothelial MV release is still a matter of debate (94,95). Our group has demonstrated that annexin I/phosphatidylserine receptor-dependent endothelial MV incorporation by ECs protects endothelial and endothelial-regenerating cells against apoptosis (96). Inhibition of p38 activity by annexin I-containing endothelial MV is possibly involved in endothelial MV-mediated protection. However, whether annexin I also mediates endothelial EV uptake in vivo will be explored in additional animal models. Another study showed that platelet-derived MVs induced changes in the early outgrowth cell, secretome, toward a more proangiogenic profile and amplified the early outgrowth cell-mediated induction of endothelial regeneration in vitro and in vivo (97). These studies indicate that MVs may influence the endothelial regeneration by the following 2 mechanisms: they

FIGURE 2 Detrimental Effects of Extracellular Vesicles on Vascular Function



The functional effect of EVs in cardiovascular disease is extremely complex and depends on the cellular origin, the functional state of the releasing cells, the biological content, the distinct recipient cell, and the transfer capacity of intravesicular functional bioactive molecules. Here, we illustrate the role of EVs as active promoters of endothelial dysfunction, vascular calcification, atherogenesis, plaque instability and thrombosis. Endothelial EVs impair vasorelaxation through local oxidative stress or through increased NADPH oxidase activity. EVs released by erythrocytes react with and degrade NO, EVs from platelet and atherosclerotic plaque induce endothelial apoptosis, both mediating endothelial dysfunction. Monocyte-derived EVs transfer inflammatory miRNAs into endothelial cells inducing vascular inflammation. Vascular smooth muscle cell-derived EVs act as mediators of vascular calcification modulating atherogenesis. EVs isolated from atherosclerotic plaques transfer ICAM-1 to endothelial cells and recruit inflammatory cells, contributing to plaque instability by promoting neovascularization. Once plaque rupture occurs, monocyte EVs and endothelial EVs initiate the coagulation cascade by the expression of tissue factor contributing to thrombosis. EV = extracellular vesicles; miRNA = microRNA; NO = nitric oxide.

could directly interact with ECs and promote vascular regeneration, or they may activate endothelial progenitor cells, facilitating endothelial repair (96,98). In line with these findings, endothelial MVs carrying endothelial protein C receptor and activated protein C (APC) could also promote cell survival by induction of cytoprotective effects (99).

Among the biological contents transferred by EVs into target cells, miRNAs seem to play a crucial role by affecting mRNA and protein expression in recipient cells (100–102). Studies by our group have shown that endothelial MVs promote vascular endothelial repair by delivering functional miRNA-126 into recipient endothelial and vascular smooth muscle cells (103,104). Of note, endothelial MV-mediated miRNA-126-induced endothelial repair was altered under pathological hyperglycemic conditions (103). These findings emphasize the fact that endothelial MVs can stimulate endothelial repair by functionally influencing migration and proliferation capacities in target cells, in addition to the already described regenerative potential of endothelial MV in interaction with progenitor cells. However, although miRNA-126 plays an important role in vascular health, miRNA-126-mediated downstream signaling and processing are not clearly identified and must be addressed in future research. Of note, EVs could activate EC by mRNA transfer from endothelial progenitor cells stimulating angiogenesis (105). In line with these findings, MVs from ischemic muscle promoted progenitor cell differentiation and subsequent postnatal vasculogenesis (106). Besides MVs, exosomes also play an important role in cardiovascular regeneration. Exosomes derived from mesenchymal stem cells reduced myocardial ischemia/reperfusion injury (107). Furthermore, exosomes from cardiac progenitor cells increased the migratory capacity of ECs in vitro and may contribute to vascular

TABLE 1 Detrimental and Favorable Effects of Extracellular Vesicles on Vascular Function

Effect	EV Type	Isolation Method	Donor Cell/Origin	In Vitro Experiment	In Vivo Experiment	Effects	Mechanisms	Ref. #
Detrimental effects								
	Injured endothelial MVs	Centrifugation 20,000 *g*	Glucose-treated HCAECs	HCAECs	ApoE$^{-/-}$ mice	Induce EC inflammation	Up-regulate ICAM-1 and VCAM-1 in EC by activating p38	(33)
	Endothelial MVs	Ultracentrifugation 100,000 *g*	RMVECs	Aortic rings from rats	–	Impair vasorelaxation	Local oxidative stress	(41)
	Circulating MVs	Centrifugation 13,000 *g*	Patients with MI	Aortic rings from rats	–	Vasomotor dysfunction	Impair endothelial NO transduction pathway	(42)
	Erythrocyte MVs	Differential centrifugation	Human packed red blood cells under standard blood banking conditions	–	Rat vasoactivity models	Reduce vasoconstrictor effects	Degrade vasodilator NO	(45)
	Platelet exosomes	Ultracentrifugation 100,000 *g*	Platelets from septic patients	ECs	–	Induce ECs apoptosis	Superoxide; NO and peroxynitrite production	(46)
	PMN MVs	Ultracentrifugation 100,000 *g*	PMNs from healthy volunteers	HUVECs	–	Induce ECs activation	Stimulate EC cytokine release Induction of tissue factor	(53)
	Oxidized MVs	Ultracentrifugation 100,000 *g*	Oxidatively modified HUVECs	Monocytes	–	Stimulate monocytes adhesion to ECs	Contain oxidized phospholipids	(56)
	Plaque MVs	Centrifugation 20,500 *g*	Human atherosclerotic plaques	HUVECs	–	Promote inflammatory response	Carry catalytically activeTNF-alpha converting enzyme (TACE/ADAM17) Enhance the processing of TNF-α and TNF receptor	(70)
	Monocyte MVs	Ultracentrifugation 100,000 *g*	Human peripheral blood monocytes	VSMCs	–	Induce VSMCs cell death	Deliver cell death message via encapsulated caspase-1	(77)
	CD40 ligand plus plaque MPs	Centrifugation 20,500 *g*	Human atherosclerotic plaques	HUVECs	Wild-type and BalbC/ Nude mice	Stimulate endothelial proliferation and angiogenesis	CD40L signaling	(82)

Continued on the next page

regeneration in vivo (108). Moreover, CD34^{+} exosomes promoted angiogenesis and preserved cardiac function in ischemic myocardium by delivery of sonic hedgehog (109).

In summary, EVs derived from endothelium, platelets, or endothelium-regenerating cells play a fundamental role by facilitating regenerative processes after vascular or myocardial injury (10,110,111).

Anti-inflammatory effects of extracellular vesicles. Several studies have reported anti-inflammatory effects of EVs. Of interest, neutrophils secrete MVs, which in turn promote anti-inflammatory release of transforming growth factor (TGF)-β1 from macrophages. These findings suggest MVs are potent anti-inflammatory effectors, which at an early stage of inflammation could contribute to its resolution (112). This effect seems to be mediated by annexin I expression on the surface of these EVs (113). EVs are also taken up by monocytes and B cells through diverse mechanisms and affect target cells toward an anti-inflammatory phenotype (114). Mesenchymal stem cells contribute to inflammatory

TABLE 1 Continued

Effect	EV Type	Isolation Method	Donor Cell/Origin	In Vitro Experiment	In Vivo Experiment	Effects	Mechanisms	Ref. #
Favorable effects								
	Endothelial apoptotic bodies	Centrifugation 16,000 *g*	HUVECs	HUVECs	Mice models of atherosclerosis	Promote atheroprotective effects Increase plaque stability Enhance progenitor cells recruitment	MiRNA-126-dependent inhibition of RGS16 Enhance CXCR4 and CXCL12	(27)
	Endothelial MVs	Centrifugation 20,000 *g*	HCAECs	HCAECs	—	Prevent HCAECs apoptosis	Annexin I/phosphatidylserine receptor–dependent inhibition of p38 activation	(96)
	Platelet MVs	Centrifugation 20,000 *g*	Platelets	EOCs	Mice models of arterial wire-induced injury	Enhance vasoregenerative potential of EOCs	Enhance EOCs recruitment, migration, differentiation Release proangiogenic factors	(97)
	Endothelial MVs	Centrifugation 20,000 *g*	HCAECs	HCAECs	Electric injury of murine carotid artery	Promote ECs migration and proliferation Accelerate re-endothelialization	Inhibit SPRED-1 via EMV-mediated transfer of miRNA-126	(103)
	Endothelial MVs	Centrifugation 20,000 *g*	HCAECs	VSMCs	Wire injury of murine carotid artery	Reduce neointima formation Diminished VSMCs proliferation and migration	Inhibit LRP6 via EMPs-mediated transfer of miRNA-126-3p	(104)
	Exosomes	Differential centrifugation	CMPCs	HMECs	—	Stimulate HMECs migration	EMMPRIN-mediated	(108)
	Exosomes	HPLC	Mesenchymal stem cells	—	Mice models of myocardial I/R injury	Reduce local and systemic inflammation	Restore bioenergetics Reduce oxidative stress Activate pro-survival signaling	(116)
	Endothelial MVs	Centrifugation 20,000 *g*	HCAECs	Monocytes	ApoE-deficient mice	Promote anti-inflammatory effects	Reduce endothelial ICAM-1 expression via the transfer of functional miRNA-222	(118)
	Endothelial exosomes	Centrifugation 20,500 *g*	KLF2-transduced or shear-stress-stimulated HUVECs	HASMCs	Aorta of ApoE knockout mice	Atheroprotection	EV-mediated transfer of miRNA-143/145	(120)
	Circulating MVs	Ultracentrifugation	Blood	VSMCs	ApoE-deficient mice	Penetrate the vascular wall Inhibit VSMCs proliferation and migration	miRNA-223-mediated IGF-1R/PI3K-Akt pathway	(121)

ApoE = apolipoprotein E; CMPC = cardiomyocyte progenitor cell; EC = endothelial cell; EMMPRIN = extracellular matrix metalloproteinase inducer; EMV = endothelial MV; HASMC = human aortic smooth muscle cell; HCAECs = human coronary artery endothelial cells; HMEC = human microvascular endothelial cell; HPLC = high-performance liquid chromatography; HUVEC = human umbilical vein endothelial cell; I/R = ischemia/reperfusion; ICAM = intercellular adhesion molecule; KLF = Krüppel-like factor; MI = myocardial infarction; MV = microvesicles; NO = nitric oxide; PMNs = polymorphonuclear leukocytes; RGS16 = regulator of G-protein signaling; RMVEC = rat renal microvascular endothelial cell; SPRED = sprouty-related EVH1 domain-containing protein; TNF = tumor necrosis factor; VSMC = vascular smooth muscle cell.

repression by releasing exosomes that induce secretion of anti-inflammatory cytokines such as IL-10 and TGF-β (115). Administration of mesenchymal stem cell-derived exosomes in a myocardial ischemia/reperfusion injury model resulted in a significant reduction of local and systemic inflammation after 24 h (116). In a renal ischemia/reperfusion model in rats, intravenously administered mesenchymal stem

cell-derived MVs limited inflammation as well as renal fibrosis (117). Finally, endothelial MVs promoted anti-inflammatory effects in vitro and in vivo by reducing endothelial ICAM-1 expression by the transfer of functional miRNA-222 into recipient cells (118). In line with these data, ECs suppressed monocyte activation through secretion of exosomes containing anti-inflammatory miRNAs (119). Importantly, despite a large amount of in vitro cellular studies, more in vivo models exploring local and systemic inflammation should be applied to validate and confirm the anti-inflammatory effects of EVs.

Plaque stabilization and antithrombotic effects. miRNA-containing EVs have been shown to promote vascular protection and plaque stabilization through various mechanisms. Injection of miRNA-126-3p-enriched apoptotic bodies of endothelial origin promoted atheroprotective effects by limiting plaque size, increasing plaque stability, and enhancing progenitor cell recruitment. miRNA-126-dependent inhibition of regulator of G protein signaling (RGS16) and subsequent enhancement of CXCR4 and CXCL12 was elaborated as the underlying mechanism (27). Exosomes from KLF-2–transduced or shear-stress–stimulated ECs are enriched in miRNAs 143 and 145. By transferring functional miRNAs, EVs were shown to control target gene expression in vascular smooth muscle target cells and reduce atherosclerotic lesion formation in the aorta of apolipoprotein E knockout mice. These findings suggest that atheroprotective stimuli induce communication between ECs and vascular smooth muscle cells through miRNA-transferring EVs (120). Similarly, circulating miRNA-223–containing exosomes could penetrate the vascular wall and inhibit vascular smooth muscle cell proliferation and migration, resulting in decreased plaque size (121). Whereas MVs are involved in enhancing blood clotting processes, exosomes seem to suppress platelet aggregation and occlusive thrombosis by inhibiting platelet CD36, inducing antithrombotic effects. However, further research is needed to validate these findings in adequate in vivo models and to understand the opposing roles of exosomes and MVs in this context. Finally, platelet-derived exosomes reduced CD36-dependent oxidized low-density lipoprotein binding and macrophage cholesterol loading, potentially contributing to atheroprotection (122). **Figure 2** illustrates the known beneficial effects of EVs in the regulation of vascular integrity. **Table 1** summarizes the most important characteristics of studies exploring the effect of EVs on vascular health and disease.

In conclusion, beneficial and detrimental effects of EVs have been described in the regulation of vascular health and disease. However, there are no constant rules showing a clear relationship between origin and function of EVs. EVs derived under pathological conditions can induce cardiovascular harm (e.g., plaque EVs promote inflammatory response [70]) but also demonstrate atheroprotective functions (e.g., MVs from ischemic muscle induce progenitor cell differentiation [106]). Even the same original EVs can show both detrimental and favorable effects (e.g., endothelial EVs impairing vasorelaxation on one hand [41] and reducing neointima formation on the other hand [104]). In order to clarify the multifaceted character of EVs and make data more comparable, additional efforts should be put into standardized EV generation techniques. Once they are established, in-depth exploration of EV-incorporated and transferred biological molecules and their intracellular processing is necessary to gain more clarity in the understanding of EV function.

THERAPEUTIC POTENTIAL OF EXTRACELLULAR VESICLES IN CARDIOVASCULAR DISEASES

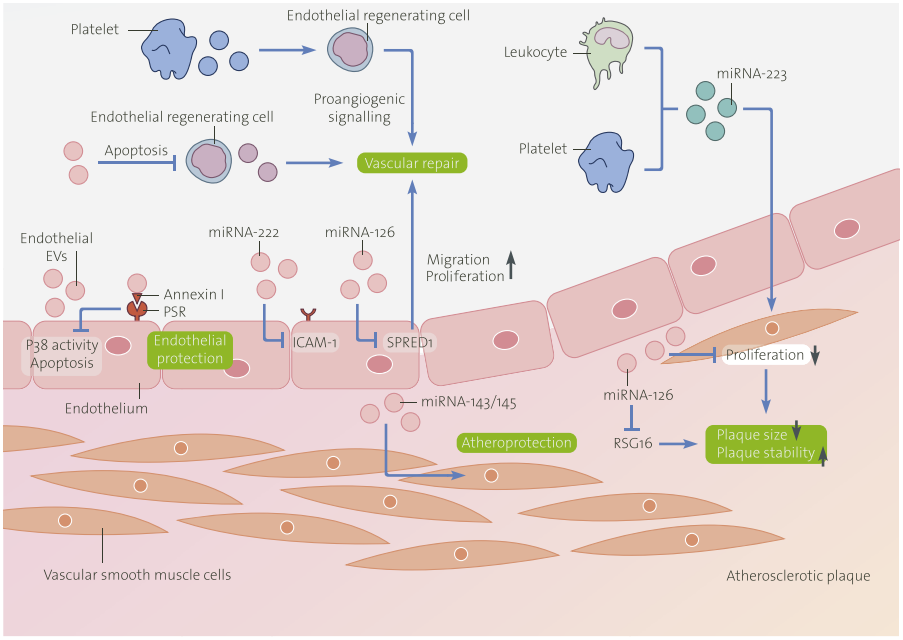
EXTRACELLULAR VESICLES AS NOVEL THERAPEUTIC TOOL?

EVs have emerged as vectors for transferring biological information by proteins or genetic material, thereby maintaining vascular homeostasis, favoring endothelial repair, or even limiting atherosclerosis.

Due to these beneficial effects, there has been a rising interest in the potential use of EVs as therapeutic vectors in the field of cardiovascular medicine and regenerative therapy. Multiple studies have shown that the transfer of functional miRNAs into target tissue by EVs promotes vascular regeneration and atheroprotection (27,103,119,120,123), highlighting the therapeutic potential of miRNA-transferring EVs. In addition to miRNA-containing EVs, many reports describing nanoparticles as a new approach to transport miRNAs or anti-miRNAs to recipient cells have been published recently (124–126) (**Figure 4**).

Chen et al. (124) developed miRNA-34a–containing liposome-polycationhyaluronic acid (LPH) nanoparticle for systemic delivery of miRNA-34a into lung metastasis of murine melanoma, resulting in significant down-regulation of surviving expression in the metastatic tumors, as well as reduced tumor. Furthermore, biodegradable polymer nanoparticles coated with cell-penetrating peptides for an effective delivery of chemically modified oligonucleotide

FIGURE 3 Beneficial Effects of Extracellular Vesicles on Vascular Function

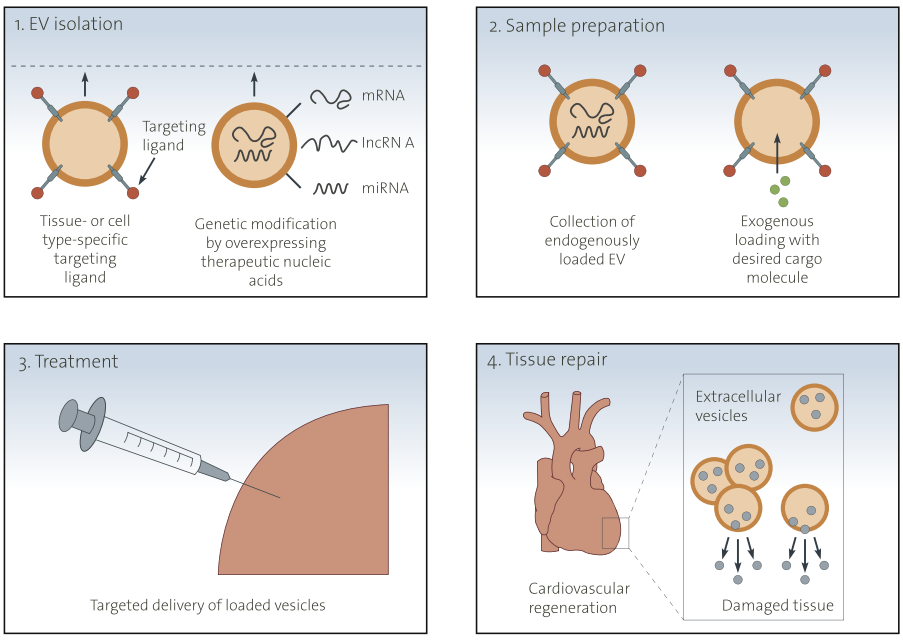


An increasing body of evidence points out the beneficial influence of certain EVs of diverse cellular sources in cardiovascular biology. This figure illustrates the favorable effects of EVs on endothelial and vascular function, atherosclerosis, and plaque stabilization. Endothelial EVs reduce endothelial apoptosis by inhibition of p38 activity mediated by an annexin I/phosphatidylserine receptor-dependent mechanism, contributing to endothelial protection. Moreover, endothelial EVs decrease endothelial regenerating cell apoptosis, facilitating endothelial repair. Platelet-derived MVs induce alterations in the endothelial-regenerating cell secretome toward a more proangiogenic profile and amplify vascular protection. Among the biological content transferred by EVs into target cells, miRNAs play a crucial role. Endothelial EVs promote vascular endothelial repair by inhibition of SPRED1 by delivering functional miRNA-126. Endothelial EVs promote anti-inflammatory effects by reducing endothelial ICAM-1 expression by the transfer of functional miRNA-222 into recipient cells. Exosomes from KLF-2-transduced or shear-stress-stimulated endothelial cells attribute to atheroprotection by transferring miRNA-143/145. Circulating leukocyte- and platelet-derived miRNA-223-containing exosomes penetrate the vascular wall, inhibit vascular smooth muscle cell proliferation and migration, resulting in decreased plaque size. Endothelial apoptotic bodies decrease vascular smooth muscle cell proliferation, limit plaque size, and increase plaque stability by miRNA-126-dependent inhibition of G-protein signaling (RGS16) pathway. MV = microvesicle; other abbreviations as in Figures 1 and 2.

analogues have been described. This nanoparticle system was used to block the activity of the oncogenic miRNA-155, as well as to attenuate the expression of the proto-oncogene Mcl-1, leading to reduced cell viability and pro-apoptotic effects in the recipient cells (127). Another approach targeting miRNA-155 described decelerated tumor growth after application of polymer nanoparticles containing antisense peptide nucleic acids with subsequent miRNA-155 inhibition (125). An integrin $\alpha v\beta 3$-targeted nanoparticle was used by Anand et al. (128) to deliver anti-miRNA-132 to the tumor endothelium of human breast carcinoma in mice, causing restored p120RasGAP expression in the tumor endothelium, thereby suppressing angiogenesis and decreasing tumor burden. Although these studies focused on miRNA or anti-miRNA delivery using nanoparticles mainly as a therapeutic tool to combat cancer, it is reasonable that nanoparticles can also be used to deliver miRNAs to recipient vascular cells for tackling inflammation and development of atherosclerosis (129). In this context, magnetic nanoparticle-assisted (circumferential) gene transfer into the vascular endothelium has recently been described as a promising novel strategy to transfer biological messages into the diseased vasculature (130–132).

TRANSLATION INTO CLINICAL USE. EVs have multiple advantages over currently available drug delivery vehicles, such as their ability to overcome natural barriers, their intrinsic cell-targeting properties, protection of their biological cargo from degrading enzymes, and stability in the circulation (133). EV subpopulations could be used as a cargo

FIGURE 4 Potential Therapeutic Use of Extracellular Vesicles



Potential therapeutic application of extracellular vesicles includes the following 4 critical steps: 1) Extracellular vesicles can be modified by using tissue- or cell-type-specific ligands present on their surface. Endogenously expressed molecules such as miRNA and noncoding RNAs can be genetically engineered for therapeutic use (e.g., genetic modification by overexpression therapeutic nucleic acids). 2) Exogenous loading permits the collection of extracellular vesicles with desired cargo molecules. The collection and purification of extracellular vesicles can be carried out by various methods, including differential ultracentrifugation, ultrafiltration, sucrose gradient centrifugation, or immunoprecipitation. 3) Extracellular vesicles, loaded by any of these strategies, can be delivered into target cells or tissues with different delivery methods (e.g., intravenously injection or intracellular injection). 4) The loaded vesicles can function as favorable effectors in intercellular vascular signaling, contributing to the cardiovascular regeneration in damaged tissue.

system for efficient and selective drug delivery to a distinct cell type within diseased tissues. This approach offers the additional advantages of low immunogenicity because patient-derived tissue could be used as the source of individualized and biocompatible drug delivery vehicles (134,135) (**Figure 3**).

Interestingly, tumor cells incubated with chemotherapeutic drugs are able to package these drugs into EVs, which can be collected and used to effectively kill tumor cells in murine tumor models without typical side effects (136). Moreover, tumor cell-derived EVs were used as a unique carrier system to deliver oncolytic adenoviruses to human tumors, leading to highly efficient cytolysis of tumor cells. These findings highlight a novel adenovirus delivery system with promising clinical applications (137).

An important issue regarding EV therapeutics is the biodistribution of EVs. Intravenously injected EVs are of particular interest in the treatment of cardiovascular alterations, as the entire vascular network would be exposed to EVs. However, to selectively direct EVs to target cells or tissue, cell-specific ligands must be stably expressed on the surface of EVs. Using an innovative approach of donor cell engineering with target cell-specific ligand expression resulted in targeted delivery of short interfering RNA (siRNA) and miRNA-loaded EVs to target neurons (138) and breast cancer cells (139).

Despite promising perspectives for the treatment of cardiovascular pathologies, EV-based therapies still need more investigation to translate experimental data into clinical application. One challenge would be to control the fragile equilibrium between the harmful and beneficial effects reported for EVs in the context of CVD. Furthermore, the off-side effects and clearing mechanisms of EVs need to be better explored before they can be seriously considered as a novel therapeutic tool for combatting CVD (140).

STUDY LIMITATIONS AND METHODOLOGICAL OBSTACLES IN EV RESEARCH. Despite the emerging role of EVs as regulators of health and disease, there

TABLE 2 Overview of Commonly Used EV Isolation Methods

Method	Principle of Separation	Advantages	Disadvantages	Ref. #
UC	Size and density	Widely used	Relatively long procedure Low throughput Depends on viscosity of biological fluids	(143)
DG	Size and density	High purity of EVs	Time-consuming	(145)
Ultrafiltration	Size	Time efficient Effective to concentrate EVs	Low purity of EVs	(146)
Precipitation kits	PEG-mediated	High yield Rapid	Low purity of EVs	(142,147)
SEC	Size	Quick procedure Reproducibility	Low purity of EVs	(143,148)
Affinity capture	Binding with EVs surface components	Production of subpopulations of EVs Relatively high purity	High cost (antibody-based) May damage surface components of EVs	(143)

DG = density gradient; PEG = polyethylene glycol; SEC = size-exclusion chromatography; UC = ultracentrifugation; other abbreviations as in Table 1.

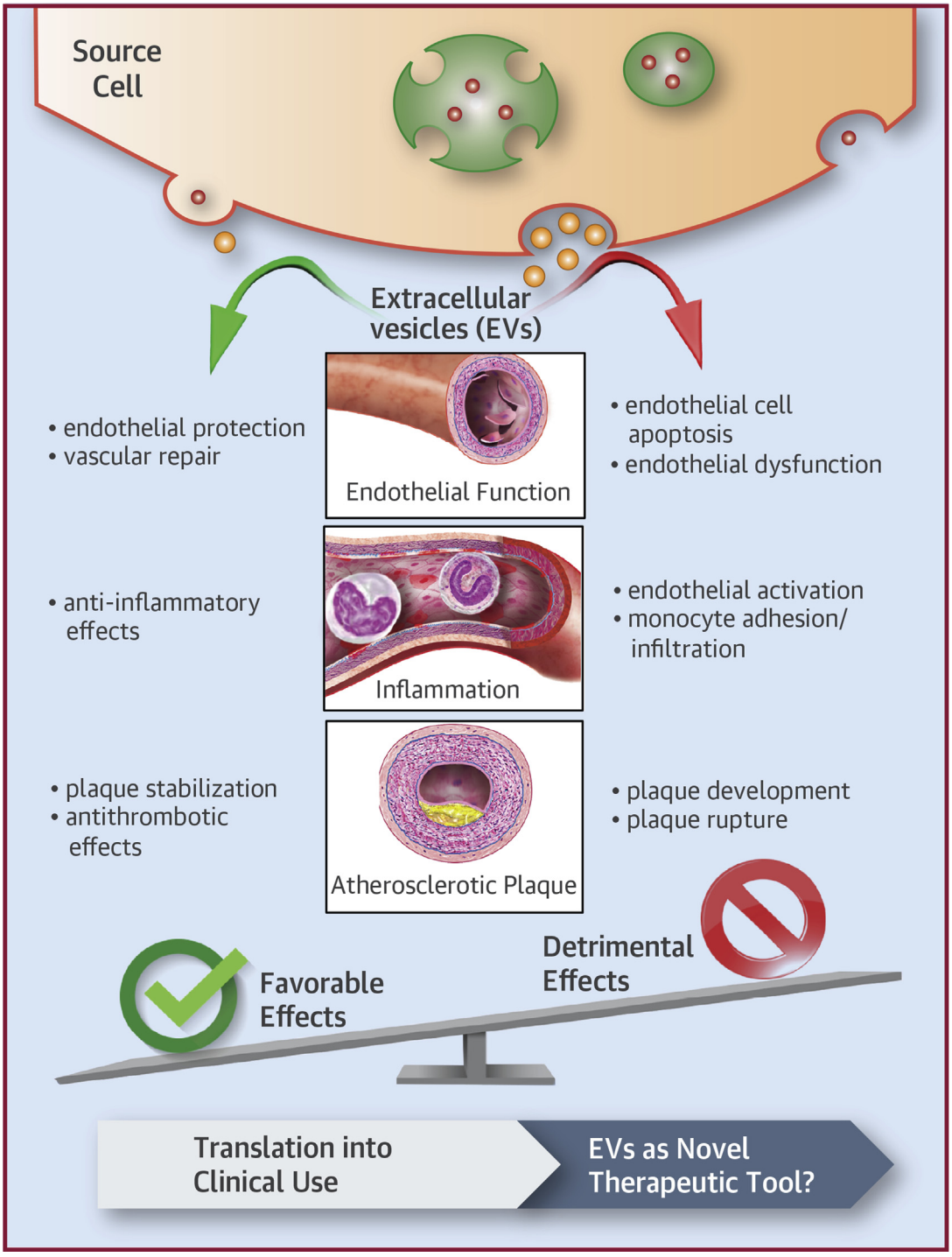
are still general limitations in EV research. Within this paragraph, we highlight relevant obstacles, which need to be addressed to better understand EV functions and move the EV field forward.

Methodological obstacles in isolating EVs. Isolation and purification of EVs vary between different research groups and also depend on the donor cells from which they are derived. Therefore, EV classification, isolation, and purification needs to be standardized to ensure that EV analysis is reproducible and internationally comparable among different research groups (141). Furthermore, within EV populations, many distinct subtypes of vesicles exist. However, the currently used methods (differential ultracentrifugation, polymer-based precipitation, density gradients, microfiltration, size-exclusion-based approaches, or polyethylene glycol [PEG]-mediated isolation techniques) all have different pros and cons in attempting to isolate pure EVs with a distinct size and surface markers (142). The most commonly used isolation methods are differential centrifugation, followed by ultracentrifugation. Two major problems with these techniques are the relatively long procedure times and low throughput, limiting their application in the clinical setting (143). Moreover, the yield depends on the viscosity of biological fluids, so the samples with a relatively high viscosity such as plasma would significantly reduce production (144). Density gradient isolation can promote both the yield and purity of EVs compared with ultracentrifugation; however, the procedure is time-consuming and hard to standardize (145). Ultrafiltration is time-efficient and can concentrate EVs up to 240-fold, but the low purity of EVs is an obstacle (146). Commercial EV precipitation kits are based mainly on PEG. Although the PEG-mediated technique provides a high-yield, rapid, and inexpensive EV isolation method from both culture media and body fluids, some other contaminants are also co-purified, leading to the low purity of EVs (142,147). In the clinical setting, size-exclusion chromatography and affinity capture are the 2 methods most often applied to isolate EVs. Size-exclusion chromatography can remove most soluble components and is a relatively quick procedure with good reproducibility (148), but it also faces the possible problem of protein or RNA contamination (143). Compared with other methods, affinity capture can produce subpopulations of EVs with relatively high purity, but the cost of preparation (antibody based) may limit its applicability and may damage surface proteins and functionality of EVs (143). Therefore, the development of new, more selective isolation techniques is urgently needed to increase the purity of each vesicle subpopulation (149). The pros and cons of each available method are summarized in Table 2.

Size measurement techniques for EV characterization. Size is an important defining property of EVs, and measurement of diameter to determine a size distribution is a critical step for EV studies. Size distribution measurement technologies include electron microscopy (EM), flow cytometry (FC), nanoparticle tracking analysis, resistive pulse sensing, and atomic force microscopy (AFM). Although these techniques are commonly used in practice (142), some issues are still unsolved. First, the ideal size measurement should detect EVs with a diameter of 50 nm and larger (150), but most methods, except for EM, cannot detect the smallest EVs (151). Second, the results of size distribution, even for the same EV subpopulation,

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 2, NO. 6, 2017
DECEMBER 2017:790–807

CENTRAL ILLUSTRATION **Extracellular Vesicles as Regulators of Vascular Health and Disease**



Jansen, F. et al. J Am Coll Cardiol Basic Trans Science. 2017;2(6):790–807.

Many types of cells release EVs, such as exosomes and microvesicles, by different mechanisms. EVs have both favorable and detrimental effects on vascular integrity. The use of genetically modified EVs might represent a novel therapeutic tool in the field of cardiovascular medicine and regenerative therapy. EV = extracellular vesicles; miRNA = microRNA.

may show different results depending on the method used (152). Furthermore, there are no standard protocols (e.g., optimal EM-EV measurement protocols) (142). Importantly, some methods, such as nanoparticle-tracking analysis or resistive pulse sensing, cannot distinguish membrane vesicles from nonmembranous particles of similar size, so the results should be compared by using EM, AFM, or other microscopy technique (153).

Regulation of EV packaging. Although some key players in sorting of cargoes into EVs are revealed (e.g., sumoylated hnRNPA2B1 is reported to control the location of miRNAs into exosomes through binding to specific motifs [154]), the underlying mechanisms mediating the packaging and loading of selected molecules into EVs remain largely unknown. Therefore, additional studies exploring EV biogenesis and mechanisms regulating EV packaging are important to understand cardiovascular injury and repair induced by EVs (155).

Methodological issues on EV uptake experiments in vitro. EV uptake experiments are usually performed by direct visualization. Therefore, fluorescent lipid membrane dyes, such as PKH26 (156), PKH68 (157), or rhodamine B (158), are used to stain EV membranes. One potential issue with membrane-binding dyes is that fluorescent molecules could potentially affect the uptake and biological behavior of EVs. However, EV incorporation has been observed with many different lipid-binding dyes, suggesting that such molecules do not affect internalization of vesicles; nevertheless, additional studies are needed to verify whether the biological behavior of EVs is affected by dyes. Another potential limitation of the use of lipophilic dyes is leaching of the fluorescent molecules from EVs into cellular membranes, potentially leading to a pattern of internalization that is due to normal membrane recycling rather than EV uptake. However, direct measurement of the fluorescence transfer rate between EVs and recipient cells support the idea that the increased fluorescence in cells is due to specific uptake of EVs rather than nonspecific dye leaching (159). Another issue which must be considered is the fact that most EV uptake studies have relied on fluorescence microscopy, which has limited resolution because the wavelength of visible light is approximately 390 to 700 nm; therefore, single EVs or aggregated vesicles, which are <390 nm in diameter, cannot be distinguished. This should not affect the assessment of EV uptake in general but may affect the visualization and dynamic localization analysis of individual EVs. Nevertheless, the increasing use of confocal microscopy has confirmed that EVs smaller than 390 nm such as exosomes can be incorporated into recipient cells (160).

Lack of EV secretion and uptake models in vivo. The secretion of EVs by parent cells and uptake by recipient cells are precisely regulated. However, few data are available studying concrete mechanisms regulating MV release and clearance. Moreover, physiological models are not well established (e.g., considering time-dependent release kinetics of EVs from parent cells). Finally, the absence of adequate in vivo models to explore the generation and uptake of cell-specific EVs limits the experimental opportunities to study EV functions in vivo.

Unclear processing of EVs and their content after cellular uptake. EVs interact with recipient cells by transferring their biological contents through membrane fusion in a ligand-receptor-mediated way or by endocytosis, pinocytosis, or phagocytosis (12) (**Figure 1**). However, processing of incorporated EVs and their intravesicular content after cellular uptake is entirely unknown. To address this point, tracking experiments using fluorescence labeleling represent a possible option to gain further insight into time-dependent cellular processing mechanisms of biological contents transferred by EVs (161). Nevertheless, stable labeling of intravesicular contents is highly demanding technically, and further efforts are needed to improve these techniques (162).

Taken together, there are still serious methodological issues that need to be addressed. Importantly, recent position research papers from the International Society of Extracellular Vesicles have made a first step in the right direction to standardize EV analysis internationally among different laboratories (141,147,153). In addition, EV-TRACK (163), a novel crowd-sourcing database, has recently been implemented, centralizing knowledge about EV biology and methodology with the goal of stimulating authors, reviewers, editors, and funders to put experimental guidelines into practice (164).

CONCLUSIONS

In the regulation of cardiovascular health and disease, EVs act as urgent effectors by transferring bioactive molecules into adjacent and distant recipients. EV-mediated intercellular vascular signaling results in detrimental and favorable effects on vascular integrity. Studies illustrate that EVs can contribute to atherosclerosis development and progression. In contrast, EVs also emerge as crucial regulators of vascular homeostasis and mediate

vascular protection. Finally, the use of genetically modified EV might represent a novel therapeutic tool in the field of cardiovascular medicine and regenerative therapy (**Central Illustration**).

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KEY WORDS cardiovascular disease, extracellular vesicles, microvesicles