Cardiovascular disease (CVD) is the number one cause of death globally. Figures from the World Health Organization show an estimated 17.3 million deaths from CVD in 2008 alone, with a predicted increase to ≈25 million deaths per annum by 2030. Although mortality from CVD has declined in the United States over the past 10 years, there is still an average of 2150 Americans who die from CVD each day. Myocardial infarction remains a major cause of mortality and morbidity. The restoration of blood and oxygen to the ischemic myocardium under threat of infarction is of paramount importance, but reperfusion paradoxically exacerbates the cellular damage incurred during severe ischemic insult. Although modern acute coronary care has significantly improved survival rates after a heart attack, the sequelae of ischemia and reperfusion injury frequently include hypertrophy and heart failure. In response, the heart may undergo various metabolic and structural adaptations, including the growth of new vessels (angiogenesis). Factors that can influence or interrupt either the initial damage or the pathological response are being actively sought as novel means of treatment. Ideally, such treatments should be safe, effective, specific, and, for ease of delivery, noninvasive or minimally invasive.

Exosomes are nanosized lipid vesicles released from cells. They are capable of transferring proteins, mRNA, and miRNA between cells and, therefore, represent a potential means of intercellular communication. Exosomes can be proangiogenic and may have cardioprotective properties. In contrast, their larger cousins, microvesicles, seem to have generally detrimental effects that are prothrombotic and proinflammatory. Exosomes are released from multivesicular bodies via an exocytic pathway and have the potential for cell-specific targeting. This normal process is hijacked during various pathological conditions, such as cancer, viral infection, and amyloidopathies. We assess the evidence for a role of exosomes and microvesicles in normal cardiovascular physiology, as well as during cardiovascular disease. In addition to offering a potential source of cardiovascular biomarkers, exosomes may offer a nonimmunogenic means of manipulating the heart. (Circ Res. 2014;114:325-332.)

Key Words: cardiovascular diseases ■ exosomes ■ heart

Circulatory disease involves blood vessels and the heart. Figures from the World Health Organization show an estimated 17.3 million deaths from CVD in 2008 alone, with a predicted increase to ≈25 million deaths per annum by 2030. Although mortality from CVD has declined in the United States over the past 10 years, there is still an average of 2150 Americans who die from CVD each day. Myocardial infarction remains a major cause of mortality and morbidity. The restoration of blood and oxygen to the ischemic myocardium under threat of infarction is of paramount importance, but reperfusion paradoxically exacerbates the cellular damage incurred during severe ischemic insult. Although modern acute coronary care has significantly improved survival rates after a heart attack, the sequelae of ischemia and reperfusion injury frequently include hypertrophy and heart failure. In response, the heart may undergo various metabolic and structural adaptations, including the growth of new vessels (angiogenesis). Factors that can influence or interrupt either the initial damage or the pathological response are being actively sought as novel means of treatment. Ideally, such treatments should be safe, effective, specific, and, for ease of delivery, noninvasive or minimally invasive.

Exosomes are extracellular lipid bilayer vesicles that range from 30 to 100 nm in diameter. They arise within endosomal compartments called multivesicular bodies, which bud internally to form intraluminal vesicles. On fusion with the plasma membrane, multivesicular bodies release their contents into the extracellular fluid, at which point the vesicles are referred to as exosomes (Figure 1). Secreted exosomes have been isolated from numerous cell lines as well as most body fluids, including saliva, urine, and plasma. Although originally ignored as cell debris, it is increasingly evident that exosome release is regulated and occurs via an energy-dependent pathway. Exosomes are believed to ferry proteins, mRNA, and miRNA cargos through the bloodstream and other body fluids, shielding them from enzymatic degradation—a process that some retroviruses may hijack to travel beneath the immune system’s radar. With the recent discovery that exosomes can deliver their cargos to recipient cells, it has become apparent that they represent a potential mode of intercellular communication throughout
found that microvascular obstruction correlated with intracoronary levels of microparticles originating from both endothelial cells and platelets. Microvesicles do have some practical advantages over exosomes in that they are more straightforward to isolate, are relatively easy to quantify using flow cytometry, and contain significantly more protein per vesicle (due to their size). This makes microvesicles an appealing area in which to find for novel biomarkers. In contrast, exosomes are below the detection limit of flow cytometry and require specialized equipment, such as nanoparticle tracking or light scattering, for their quantification. However, exosomes represent a totally distinct population of vesicles from that of microvesicles and may provide unique information on cellular status—healthy or otherwise. Also, although research is in the early stages, exosomes seem to possess characteristics making them preferable over microvesicles as novel means of delivering therapeutics.

### On the Optimal Method of Exosome Purification

A hot topic in the field of exosome research remains the optimal method of exosome purification. Consequently, this topic deserves a brief discussion here. There are essentially 4 main approaches to purification of exosomes, which are based on immune affinity capture, size filtration, size exclusion, or ultracentrifugation. Although immune affinity is regarded as having the advantage of specificity when an appropriate epitope is available, yields are often quite low. In contrast, filtration through a series of filters down to 100 nm pore size followed by centrifugation to concentrate, although yielding relatively high protein content, risks impurity because of the fragmentation of larger microparticles into smaller vesicles under filtration pressure. This may explain why the product of platelets from septic patients purified in such a manner was found to worsen cardiac function in isolated muscles. The use of ultrafiltration has been less well-explored, but methods such as cross-flow filtration are an efficient way to concentrate exosomes away from smaller protein contaminants while avoiding the hazards of passage through small apertures at high pressure. The most generally accepted method is to use a well-defined series of serial centrifugation steps that remove cells and microvesicles, followed by concentration by ultracentrifugation and subsequent density gradient purification. Although a consensus on optimal sample collection, isolation, and analysis has not yet been achieved, the International Society for Extracellular Vesicles (ISEV) has published a comprehensive list of issues to consider and a preliminary set of recommendations. It is worth noting that the optimal method for microvesicle purification is no more well-established, although efforts to standardize the method of collection, centrifugation, and transport are being made.

### Exosome Content

Given the variety of methods used to isolate exosomes, the characterization of exosome population used is essential before their analysis. Proteomic analysis of exosomes has identified common characteristic proteins on their surface and in their lumen. Typical exosomal marker proteins include the tetraspanins CD9, CD63, and CD81, and heat shock proteins such as HSP70 and HSP90. In addition, exosomes typically

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contain cytoplasmic proteins such as actin, annexins, and glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase and enolase. They also contain molecules involved in multivesicular body biogenesis such as Alix, TSG101, and Rab proteins, which are involved in exosome release. It should be appreciated that these exosomes could transport many as-yet-unidentified proteins and, in view of the cardioprotective effects that have been observed (described below), it may be hypothesized that some would be prosurvival. Our understanding of the mechanism of exosome biogenesis and release is evolving rapidly. Just a few years ago, evidence favored the release of exosomes by a mechanism independent of the endosomal sorting complex required for transport (ESCRT) machinery, but requiring the sphingolipid ceramide. 23 With the recent description of a role for syndecan, syntenin, Alix, and ESCRT’s in the control of exosome formation, 22 the balance of evidence is now swinging toward support of an ESCRT-regulated mechanism of membrane budding of exosomes. 24,25

A scale diagram using representations of crystallographic structures of some major exosomal molecules puts into perspective just how restricted the space is within exosomes (Figure 2). However, en masse, exosomes might deliver a significant quantity of proteins to effect changes in recipient cells. Experiments have shown that exosomes can transfer signaling ligands such as those of the Notch family from tumors to endothelial cells in quantities sufficient to alter their morphology. 26

Some of the proteins mentioned are already known to influence cardioprotection. The relationship of heat shock protein and cardioprotection was well-established in the 1990s. 27 Many heat shock proteins, including αB-crystallin, HSP60, and HSP70, are secreted in exosomes 28,29 and, in some instances, can be transferred to adjacent cells to confer protection against oxidative stress. 30 Interestingly, circulating HSP70 levels are negatively correlated with symptoms of CVD. 31 suggesting that exosomal HSP70 may be beneficial. HSP60 also secreted from cardiomyocytes in exosomes, 32 although the implications of this are unclear, and circulating HSP60 levels have been associated with autoimmune disease. 33 Other secreted proteins implicated in CVD or myocardial ischemia and reperfusion have been identified in exosomes. For example, the inflammatory cytokine tumor necrosis factor-α, which can induce contractile dysfunction, hypertrophy, fibrosis, and cell death, has been detected in exosomes released from hypoxic cardiomyocytes. 33 Interestingly, the tumor necrosis factor receptor has also been identified in plasma exosomes. 34 Other molecules important in the cardiovascular system identified in exosomes include proliferator activated receptor-γ, 35 phosphatase and tensin homolog, 36 annexins, 4 dipeptidyl peptidase-4, 4 epidermal growth factor receptor, 37 and a host of metabolic enzymes. 4 The p22 and gp91 subunits of phagocyte-like NADPH oxidase as well as NADPH oxidase activity have been detected in exosomes derived from platelets of septic individuals, although the significance of this is not known. 38 A recent high-profile publication demonstrated the evolutionarily conserved role for exosomes in the secretion of Wnt proteins, showing that presentation of Wnt on exosomal surfaces contributes to biological Wnt signaling. 39 The Exocarta database 40 catalogues proteins that have been identified in exosomes and also the number of studies in which they have been identified 4 as an important means of assessing the robustness of this localization. However, it is important to keep in mind that the majority of studies thus far have been performed on exosomes released from malignant cells, which may have an abnormal composition.

Plasma exosomes and microvesicles both originate primarily from platelets and megakaryocytes and, to a lesser degree, from endothelial cells, erythrocytes, and leukocytes. 10,12,13 The exosomal proteome differs according to the type of cell of origin, 4 such that platelet or endothelial exosomes can be identified by their expression of typical cellular markers such as CD31 (platelet endothelial cell adhesion molecule-1) or CD62P (P-selectin), respectively. 10 Furthermore, studies indicate that cellular stress can alter exosomal protein and RNA content, 31 suggesting the intriguing possibility that exosomes represent a snapshot of the physiological state of the cell—a kind of status update—released by cells into the circulation.

In some respects, it is easier to envisage how the transfer of even minute quantities of miRNA might have more dramatic effects compared with the delivery of proteins. Hence, the discovery that mRNA and miRNA are also localized within exosomes has generated much interest, although the extent to which plasma miRNA is contained within exosomes is still controversial. Some reports suggest that exosomes contain the majority of plasma miRNA, 42 whereas others suggest that plasma miRNAs are mainly found in complexes with carrier proteins such as argonaute 43 or high-density lipoproteins. 44 In any event, numerous studies have demonstrated the ability of exosomes (and microvesicles) to transfer miRNA to recipient cells. 25,46 In a seminal article in the field, exosomes from mast cells were shown conclusively to contain both miRNA and mRNA and to transfer miRNA to recipient cells for translation into new proteins. 45 Importantly, the RNA was protected from treatment with RNase or trypsin, so it must have been...
contained within the exosomes.\textsuperscript{45} The miRNA or mRNA content of exosomes also seems to reflect the cell they originate from. The miRNA content of dendritic cell exosomes varies with cell maturation.\textsuperscript{47} Similarly, exosomes from mast cells exposed to oxidative stress contain different mRNAs than those from control cells.\textsuperscript{48} Furthermore, exosomes from these stressed cells conferred resistance against oxidative stress to recipient cells.\textsuperscript{49} Another fascinating report has described the use of purified exosomes as an in vivo transfection reagent for the delivery into the brains of mice of miRNA, which had been loaded into the exosomes by electroporation.\textsuperscript{50}

Exosomal miRNA content may be relevant to CVD. Patients with acute myocardial infarction have increased serum levels of miR-1 and miR-133a, whereas in vitro experiments suggest that exosomes from cardiac cells can release miR-133a and transfer it to recipient cells, where it modulates gene expression.\textsuperscript{50} Curiously, miR-133a normally suppresses hypertrophy by restraining the expression of inositol 1,4,5\textsuperscript{′}-triphosphate receptor II calcium channel. Cellular levels of miR-133a have been found to decrease during hypertrophic response to pressure overload.\textsuperscript{51} Clearly, much work remains to be done to clarify the role of exosomes and miRNA in CVD.

In addition to proteins and RNA, exosomes seem to be enriched in sphingomyelins, although the levels of cholesterol and phosphatidyl cholines seem to depend on cellular origin.\textsuperscript{52} They can contain bioactive lipids such as phospholipases, arachidonic acid, and derivatives such as prostaglandins,\textsuperscript{53} which may exert effects related to inflammation.

**Exosomes and Cardioprotection**

The early promise of stem cells in cardiac regeneration generated much excitement for their potential to improve function by differentiating into new cardiomyocytes. Despite some waning of this initial optimism, improvement of cardiac function and survival is consistently observed after the injection of stem cells, apparently due to their release of paracrine factors. An intriguing possibility is that some of these paracrine effects may be mediated by exosomes. For example, exosomes purified from the conditioned medium of CD34\textsuperscript{+} stem cells are proangiogenic both in vitro and in vivo.\textsuperscript{54} Similarly, recent evidence suggests that human embryonic stem cell–derived mesenchymal stem cells (MSCs), rather than differentiating into cardiomyocytes when injected into recipient hearts, actually mediate their cardioprotective properties by the paracrine release of exosomes.\textsuperscript{55} The highly purified protective component from MSC-conditioned medium was found to contain vesicles of the same density, size, and appearance by electron microscopy as exosomes, and they expressed exosomal marker proteins (CD9, CD81, Alix).\textsuperscript{55} Exosomes were highly cardioprotective when introduced either into isolated perfused rat hearts or intravenously into anesthetized mice immediately before reperfusion. The mechanism by which exosomes confer cardioprotection seems to involve the activation of the same cardioprotective kinase pathways as preconditioning.\textsuperscript{55–58} Furthermore, the cross-species cardioprotection that is observed points to an evolutionarily conserved pathway.

Exosomes have also been isolated from in vitro cultured murine cardiac progenitor cells, and recent data suggest that they are equally able to protect the myocardium from ischemia/reperfusion injury after intramyocardial delivery.\textsuperscript{59} On a practical note, in experiments such as these, it is important that cells are cultured in serum that has been depleted of exosomes (by ultracentrifugation, for example). Second, it is essential to compare cardioprotection against vehicle containing any carrier retained during purification because, from our experience, vehicles such as bovine serum albumin or polyethylene glycol at high concentration can cause an apparent decrease in infarct size. The question now being pursued in these studies is the identity of cardioprotective ligands.

Camussi et al have undertaken extensive work demonstrating that microvesicles released by MSCs or endothelial progenitor cells can prevent acute kidney injury when injected intravenously after kidney ischemia.\textsuperscript{60,61} Interestingly, the isolation protocol used is similar to the standard ultracentrifugation protocol for exosome purification and may result in a mixture of microvesicles and exosomes. These results highlight the importance of careful characterization and definition of the population of vesicles after isolation. Microvesicles isolated from ischemic mouse hind limbs have also been found to promote angiogenesis when injected into naive ischemic limbs.\textsuperscript{62} In contrast, microvesicles purified from the plasma after a preconditioning protocol were found not to affect cardiac ischemia and reperfusion injury when injected intravenously into rats immediately before reperfusion.\textsuperscript{63} Recently, it has been suggested that plasma exosomes are cardioprotective in a Langendorff-perfused heart system.\textsuperscript{64} Given the many beneficial aspects of exosomes outlined here, their induction after hypoxia,\textsuperscript{41,65} and their ability to transmit cardioprotective signals, an attractive hypothesis is that exosomes contribute to the humoral transmission of the cardioprotective state induced by cardioprotective modalities, such as remote ischemic preconditioning. If our hypothesis is validated, then this would suggest that exosomes may harbor novel cardioprotective molecules.

The mechanism by which exosomes exert cardioprotection is almost entirely unknown. It seems to involve a direct interaction with cells in the heart, rather than blood components, because cardioprotection has been observed both in vitro and in vivo. At least in specific cases, exosomes have been demonstrated to be capable of direct transfer of RNA\textsuperscript{45–47} (Figure 3B) or protein (Figure 3A).\textsuperscript{66} For example, the Notch ligand, Delta-like 4, can be transferred between endothelial cells via exosomes.\textsuperscript{26} However, the observation that cardioprotection is rapidly induced after perfusion with exosomes suggests a more rapid mechanism than transcriptional regulation. Remarkably, exosomes/microvesicles derived from cancer cells are capable of transferring activated endothelial growth factor receptors directly from malignant cells to endothelial cells\textsuperscript{7} (Figure 3C), which then stimulate angiogenesis via autocrine production of vascular endothelial growth factor. Alternatively, proteins on the surface of exosomes may interact directly with plasma membrane receptors in the myocardium and activate their downstream intracellular signaling pathways (Figure 3D). The majority of the hundreds of G-protein–coupled receptors studied to date activate intracellular signaling pathways, which converge on PI3-kinase/Akt and ERK/MAPK, or JAK/STAT—the so-called RISK pathway or SAFE pathway of cardioprotection, respectively. Interestingly,
the paracrine cardioprotective effect of MSCs requires PI3-kinase/Akt, supporting this hypothesis.

Exosomes and CVD

Although the potential therapeutic application of exosomes in CVD is only just beginning to be explored, results thus far are exciting. Ischemic heart disease develops as a result of coronary atherosclerotic plaque formation, leading to reduced coronary blood flow. Over a period of time, coronary collateral vessels and microvascular angiogenesis develop as a response to myocardial ischemia. It is thought that angiogenesis helps preserve the functionality of ischemic myocardium. Therapeutic coronary angiogenesis and collateralization have tremendous potential as treatment strategies for patients with ischemic heart disease. There is increasing evidence for exosomes having an important role in angiogenesis. Human CD34 stem cell–derived exosomes induce angiogenic activity in vitro and in vivo. Intravenous delivery of exosomes from MSCs suppressed lung inflammation, inhibited vascular remodeling, and inhibited the development of right ventricular hypertrophy in a mouse model of hypoxic pulmonary hypertension. Exosomes from dendritic cells can modulate immune responses, and injection of exosomes derived from donor bone marrow dendritic cells was found to modulate the response to heart transplantation.

In addition to being a major risk factor for CVD, vascular complications are a major contributor to morbidity in diabetes mellitus. The potential for exosomes to shuttle miRs and proteins to the ischemic limb in peripheral vascular disease has barely been examined, but a recent study detected a significant increase in miR-15a and miR-16 levels in the serum of patients with critical limb ischemia. In addition to being conjugated to argonaute-2, the miRNAs were found within exosomes, suggesting their potential as markers of critical limb ischemia. Because miR-15a seems to inhibit angiogenesis, it will be interesting to determine whether exosomes actually contribute to ischemic injury in this case. In other situations, the injection of proangiogenic exosomes can be beneficial. For example, the previously mentioned vesicles isolated by Camussi et al have been shown to induce newovascularization in a murine model of hindlimb ischemia, stimulating angiogenesis by means of miRNA or mRNA transfer. Protection was lost after RNAse treatment or depletion of proangiogenic miR-126 and miR-296. Human cardiomyocyte progenitor cells have also been shown to release exosomes, which can stimulate the migration of microvascular endothelial cells. In an interesting recent study, human umbilical vein endothelial cells subjected to shear stress were shown to release vesicles (exosomes or microvesicles) enriched in miR-143/145, which controlled target gene expression in cocultured smooth muscle cells. Importantly, the released vesicles reduced atherosclerotic lesion formation in a mouse model of atherosclerosis.

Many of the studies performed to date used vesicles purified from cells cultured in vitro, which may have a different lipid composition, protein content, or other characteristics compared with those released in vivo. Thus, although they demonstrate a potentially useful therapeutic effect, they do not address the fundamental question of the in vivo relevance of native endogenous vesicles. This is difficult to determine without effective and specific tools to prevent microvesicle or exosome release in vivo. There are some exciting leads in this area, with the Rab proteins being implicated in exosome release; however, the exact contingent of Rab proteins involved is suspected to be cell type–specific and requires much more investigation. Similarly, the existence of a mechanism controlling the cell-specific delivery of exosomes is not well-established. A recent report suggests that selectivity of uptake is regulated by specific interactions between tetraspanins and integrins and can be controlled by altering the expression of different tetraspanins proteins.

This raises the possibility that the capacity for targeting of exosomes can be harnessed for delivery to specific cells or organs but, again, much work remains to be done in this area. This and other basic aspects of exosome biology will require much deeper understanding before their clinical application can be seriously considered.

Future Potential of Exosomes in Cardiovascular Research

Two obvious avenues for the potential exploitation of exosomes in cardiovascular field are in their use as biomarkers and in the potential for harnessing their capacity for delivery of biologics (Figure 4). A major advantage of using secreted vesicles, such as exosomes, for proteomic identification of plasma biomarkers is that simply by purifying them many possible cellular proteins of interest are separated from the morass of highly abundant serum proteins. This greatly simplifies subsequent proteomic analysis. Although research is still in the very early stages, the view is emerging that exosomes represent a snapshot of the (primarily cytosolic and plasma membrane) cellular proteome of their cell of origin. In terms of biomarker screening, a simple approach may be to analyze exosomes secreted in the urine, which may reflect protein or miRNA changes that occur with CVD. This
particularly after pressure stress. A previous study described a procoagulant microvesicle activity in exosomes from mast cells; however, exosomes produced by dendritic cells have been isolated and loaded with specific siRNA and then used to knock down BACE1, a therapeutic target in Alzheimer disease, in the brains of mice. Targeting in this case was achieved by expressing a neuron-specific peptide.

Exosomes have several characteristics that seem to make them preferable over microvesicles for the purpose of therapeutics, including lower immunogenicity. Whereas, there is evidence that some exosomes express the procoagulant protein tissue factor and may be procoagulant, for example, exosomes purified from mesenchymal-like cancer cells, by hypoxic glioblastoma cells, and bronchial epithelial cells, particularly after pressure stress. A previous study described a procoagulant activity in exosomes from mast cells; however, because these vesicles were isolated without an intermediate centrifugation step to remove microvesicles, it is likely that procoagulant microvesicles contributed to the activity observed. In contrast to these examples, exosomes from various sources, including tumors, immune cells, and body fluids, have been found to be immunosuppressive. These contrasting data emphasize once again the importance of carefully defining the vesicle population being examined and of the peril of generalizing in a field in which many of the basic characteristics are still being elucidated and defined.

Finally, a further avenue for exploitation might be in developing the means to stimulate the body’s own production and transport of exosomes that have been programmed for survival and that may be of direct benefit to patients with CVD.

Conclusions
Interest in exosomes is exploding, with more articles on exosomes published after 2011 than in all previous years combined; yet, much about their basic biology remains to be determined. By analogy with astronomy, exosomes represent a kind of dark matter of the body—invisible to direct microscopy, but whose existence can be inferred by the effects they have on other cells. The question now is, how pervasive is their influence? Certainly, they have been strongly implicated in cancer and in certain situations such as retroviral budding and transmission, but do these exosomes represent the dark side of a normal physiological process? If exosomes, present in the plasma at such extraordinary concentrations, are able to transfer protein and RNA to recipient cells in significant quantities, then they might represent a new paradigm of intercellular signal transmission. As such, exosome biology could represent a new frontier at the nanoscale, which will advance our search for novel approaches to cardiovascular signaling and cardioprotection. Although the basic aspects of exosome biology are only just beginning to be explored, it seems certain that in near future they will be generating a level of interest disproportionate to their size.

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None.

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Derek M. Yellon and Sean M. Davidson

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