ATVB in Focus
MicroRNAs: From Basic Mechanisms to Clinical Application in Cardiovascular Medicine

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Intercellular Transport of MicroRNAs

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***Abstract*—**Extracellular microRNAs (miRNA) are present in most biological fluids, relatively stable, and hold great potential for disease biomarkers and novel therapeutics. Circulating miRNAs are transported by membrane-derived vesicles (exosomes and microparticles), lipoproteins, and other ribonucleoprotein complexes. Evidence suggests that miRNAs are selectively exported from cells with distinct signatures that have been found to be altered in many pathophysiologies, including cardiovascular disease. Protected from plasma ribonucleases by their carriers, functional miRNAs are delivered to recipient cells by various routes. Transferred miRNAs use cellular machinery to reduce target gene expression and alter cellular phenotype. Similar to soluble factors, miRNAs mediate cell-to-cell communication linking disparate cell types, diverse biological mechanisms, and homeostatic pathways. Although significant advances have been made, miRNA intercellular communication is full of complexities and many questions remain. This review brings into focus what is currently known and outstanding in a novel field of study with applicability to cardiovascular disease. **(*Arterioscler Thromb Vasc Biol*. 2013;33:186-192.)**

Key Words: microRNA ■ exosomes ■ high-density lipoprotein ■ microparticles ■ extracellular RNA ■ intercellular communication

MicroRNAs (miRNAs) are small, noncoding RNAs that provide posttranscriptional regulation of gene expression and control of many metabolic and physiological processes associated with cardiovascular disease.[1–6] Most annotated genes are predicted targets of miRNAs, including many key regulators of cholesterol metabolism and cardiovascular function.[4–7] Intracellular miRNAs have proven to be critical mediators in the response to cellular stress, disease, and environmental stimuli.[8] Extracellular miRNAs are a new class of cellular messengers, as they stably exist in most biological fluids, including blood, urine, cerebrospinal fluid, saliva, semen, and breast milk.[9–16] Selectively exported and functional in recipient cells, extracellular miRNAs are now recognized as regulatory signals in cell-to-cell communication.[17–19] Very much like soluble factors, extracellular miRNAs provide intercellular gene regulation and phenotypic control. However, the delivery of miRNA cassettes or clusters of miRNAs may have a greater capacity to impact a more diverse set of genes and biological pathways than cytokines or hormones. Similar to soluble factors, extracellular miRNAs likely function to regulate gene expression in both macro and microenvironments. Here, we review the current biology of miRNAs as intercellular messengers and the phenotypic outcome of small RNA communication.

Extracellular miRNAs

Carriers of Extracellular miRNAs

Then classified as low-molecular-weight RNA, extracellular small RNAs were observed in blood as early as 2004[20]; however, miRNA profiling of human plasma or serum was not completed until 2008, when Lawrie et al[21] found miRNAs in serum of lymphoma subjects and Mitchell et al[9] found extracellular miRNAs to be stable in human plasma or serum.[9,21] Strikingly, miRNAs were reported to be functional extracellular signaling molecules in plants more than a decade ago.[22,23] Nonetheless, the global plasma miRNA signature is composed of individual subclasses of miRNA carriers. Membrane-derived vesicles, lipoproteins, and ribonucleoprotein complexes all have been found to transport extracellular miRNAs (Figure).[24–26] Exosomes and microparticles (MPs) are 2 distinct classes of membrane-derived vesicles, differentiated by their biogenesis and secretory mechanisms.[18] Exosomes, observed as vesicles ≈40 to 100 nm in diameter, are initially formed by the inward budding of the plasma membrane into multivesicular bodies within endosomes.[27] Exosomes and their miRNA content are released into the extracellular compartment on the fusion of endosomes with the plasma membrane.[18,27] Conversely, MPs are generally larger vesicles (100–4000 nm) that result from the outward budding and blebbing of the plasma membrane.[28] During apoptosis, cells can release even larger MPs or apoptotic bodies that also transport specific sets of miRNAs.[29] Originally described as platelet dust, MPs were first observed in plasma >45 years ago.[30]

Extracellular miRNAs are also transported by lipoproteins, namely high-density lipoproteins (HDL) and low-density lipoproteins (LDL), both of which are highly abundant in plasma.[26] Whereas exosomes and MPs are composed

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Table. Functional miRNAs in Intercellular Communication

miRNA	Secreting Cell	Recipient Cell	Carrier	Targeted Genes	Effect	Reference
miR-150	Monocytes	Endothelial cells	Microparticles	c-Myb	Increased migration	24
miR-223	n/a	Hepatocytes	HDL	RhoB, EFNA1	n/a	26
miR-126	Endothelial cells	Endothelial cells	Apoptotic bodies	RGS16	Antiatherosclerotic	29
miR-143	Endothelial cells	Smooth muscle cells	Exosomes/microparticles	ELK1, PHACTR4, SSH2	Antiatherosclerotic	61
miR-145	Endothelial cells	Smooth muscle cells	Exosomes/microparticles	KLF4, CAMK2d, CFL1, PHACTR4, SSH2	Antiatherosclerotic	61
miR-16	Large adipocytes	Small adipocytes	Microparticles	n/a	Lipid synthesis	63
miR-222	Large adipocytes	Small adipocytes	Microparticles	n/a	Lipid synthesis	63
miR-27a	Large adipocytes	Small adipocytes	Microparticles	n/a	Lipid synthesis	63
miR-146b	Large adipocytes	Small adipocytes	Microparticles	n/a	Lipid synthesis	63

HDL indicates high-density lipoproteins; RhoB, ras homolog gene family, member B; EFNA1, ephrin-A1; RGS16, regulator of G-protein signaling 16; ELK1, E twenty-six (ETS)–like transcription factor; PHACTR4, phosphatase and actin regulator 4; SSH2, protein phosphatase slingshot homolog 2; KLF4, krueppel-like factor 4; CAMK2d, calcium/calmodulin-dependent protein kinase type II δ chain; CFL1, cofilin 1; and n/a, not available.

of a bilayer-phospholipid shell and hydrophilic core, lipoproteins consist of a single layer of lipids, a hydrophobic core, and are defined by specific structure–function apolipoproteins. Evidence suggests that the exosomal-, HDL-, and LDL-miRNA signatures are distinct (low correlation), although some miRNAs are found in all subclasses, including miR-223.[26] Argonaute 2 (AGO2), the main functional component of the cytoplasmic miRNA ribonucleoprotein complex (miRNP), is observed bound to extracellular miRNAs, both in and out of membrane-derived vesicles.[31–33] Biophysical studies suggest that miRNAs are associated with protein complexes 50 to 300 kDa in size, which includes AGO2 as well as other ribonucleoproteins.[31,32] Recently, viral surface antigen particles were also found to transport specific miRNAs, as hepatitis B surface antigen particles were found to contain AGO2-bound miRNAs.[34]

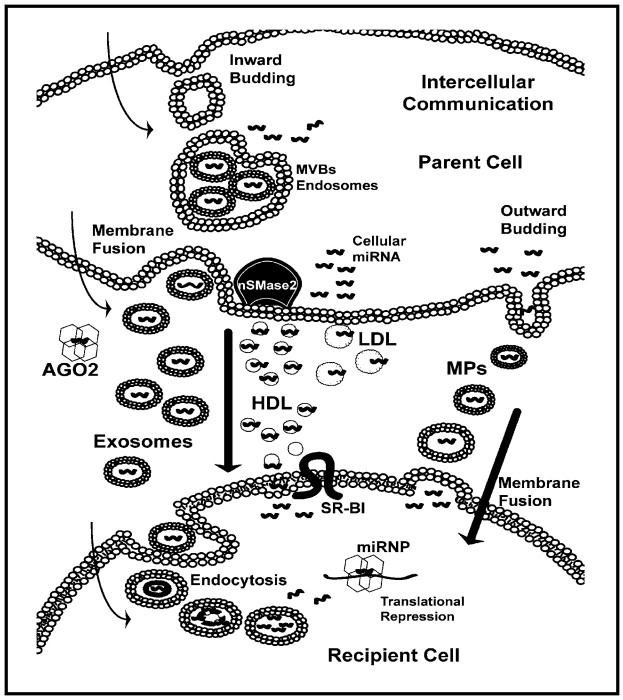

Figure. Schematic of microRNA (miRNA) intercellular communication. Secreted miRNAs are transported by extracellular carriers and transferred to recipient cells, where they alter gene expression. Membrane-derived exosomes are released from cells as endosomes containing multivesicular bodies (MVB) that fuse with the plasma membrane. Microparticles (MPs) are released from the plasma membrane through outward budding. Neutral sphingomyelinase 2 governs the release of extracellular miRNAs. Exosomes and MPs deliver extracellular miRNA through endocytosis and membrane fusion. Delivery of high-density lipoprotein (HDL)–miRNAs is dependent upon scavenger receptor B1. Argonaute 2 (AGO2) is the main structure–function protein within the miRNA ribonucleoprotein complex, where miRNAs recognize and bind to mRNA targets. LDL indicates low-density lipoproteins.

In vitro studies suggest that most cell types secrete exosomes or MPs, including neurons and inflammatory, muscle, and tumor cells.[25,35–39] However, the majority of circulating MPs and exosomes in vivo are likely secreted by platelets.[40,41] In 2002, exosomes were first reported to transfer information between cells[27,42]; however, it was not until a seminal study in 2007 that exosomes were found to contain miRNAs.[25] Differential miRNA profiles associated with membrane-derived vesicles have been described for many pathophysiologies, including cardiovascular disease.[43–45] Currently, it is unknown whether the abundances of specific miRNAs carried on extracellular protein complexes are also altered with disease. Likewise, differential HDL–miRNA signatures were observed in humans and mice with hypercholesterolemia.[26] Most interesting, many of the differential miRNAs associated with cardiovascular disease, miR-150, miR-223, and miR-92, are candidate signaling molecules, as they have each been reported to alter gene expression upon transfer to recipient cells.[24,26,46]

Cellular miRNA Export

Intercellular communication based on miRNA is composed of 3 critical processes. First, miRNAs must be selectively and actively secreted from cells and packaged into appropriate carriers. Second, miRNAs must be protected from circulating RNAses and transferred to targeted or receptor-specific recipient cells. Third and most importantly, miRNAs must retain the ability to recognize and repress mRNA targets within recipient cells. Although little is currently understood about how miRNAs are selectively exported, multiple observations suggest that the process is selective and regulated as some miRNAs are found

to be only exported and not retained in the parent cell, and specific signaling pathways have been found to regulate cellular miRNA release.[26,45,47,48–50] Most importantly, miRNA profiles of extracellular vesicles and lipoproteins are not representative of their parent cell-type, but are distinct sets of miRNAs.[24,25,47] Secondly, miRNA profiles of total plasma or serum and the individual profiles of distinct miRNA carriers in plasma or serum are surprisingly consistent among individuals.[26] Furthermore, each biological fluid compartment contains distinct miRNA signatures. Collectively, these observations support the selective export hypothesis, in that cells actively secrete specific miRNAs as a result of cellular signals or environmental cues. Extracellular vesicles released from tumor cells were found to contain miRNAs not present in the parent cell, which suggests that some miRNAs may be transcribed only to be exported.[48,49] Conversely, some cellular miRNAs may not be transcribed or processed in a given cell, but may instead be present through exogenous delivery by extracellular vesicles. Multiple studies have found that the ceramide pathway regulates the export of cellular miRNAs.[26,47,50–52] Neutral sphingomyelinase 2, the rate-limiting enzyme in the conversion of sphingomyelin to ceramide, is a critical regulator of exosome biogenesis and secretion.[53] Inhibition of neutral sphingomyelinase 2 resulted in decreased export of specific miRNAs to exosomes, but increased the export of miR-223 to HDL.[26,50–52] Previously, miR-223 was found to be enriched in nonvesicle subclasses,[24] which is supported by the observation that miR-223 is one of the most abundant and consistent miRNAs found on HDL.[26] Nevertheless, neutral sphingomyelinase 2, which resides on the inner leaflet of the plasma membrane,[54] likely serves in some capacity to govern extracellular miRNA release to differential carrier subclasses.

Transfer of Functional miRNAs

Endogenous transfer of functional miRNAs was first reported in 2009, when apoptotic bodies were found to transfer functional miR-126 to recipient cells (Table).[29] Smaller vesicles (exosomes) were found to transfer functional miRNAs in 2010, when plants cells infected by Epstein-Barr virus were reported to secrete exosomes containing viral miRNAs that altered target genes in recipient dendritic cells.[55–57] The exogenous miRNA targeting was readily differentiated from endogenous miRNA gene regulation.[57] Most importantly, these studies set forth the concept of miRNA intercellular communication. In parallel, MPs were found to transfer miR-150 to recipient endothelial cells and enhance migration.[24] This study, and others that followed, identified a novel miRNA-based communication network between inflammatory cells and the vascular endothelium. It has been proposed that extracellular miRNAs are especially adept at mediating gene regulation among cells in a microenvironment.[58] In this regard, subintimal inflammation and atherosclerotic lesions, which harbor complex multicellular microenvironments, may be a nexus of miRNA-based signaling. Multiple studies have found that endothelial cells are particularly efficient recipient cells for miRNA transfer.[24,46,59,60] However, a recent study has demonstrated that endothelial cells also secrete extracellular miRNAs, as vesicles originating from stimulated endothelial cells were found to transfer miR-143/miR-145, and alter vascular smooth muscle cell phenotype.[61]

Intercellular Communication

Cell-to-cell communication through miRNA transfer may be more perfectly suited for microenvironments, as lipoproteins and extracellular vesicles found in plasma are likely to reach many distant tissues. In addition to cardiovascular disease, many other pathophysiologies likely use circulating carriers to modulate systemic homeostasis or propagate the disease (Table). Evidence suggests that cancer likely uses circulating miRNAs and their carriers to promote tumorigenesis and malignancy.[46,60,62] Multiple reports have found that tumor cells communicate with endothelial cells through the transfer of proangiogenic miRNAs that leads to cell migration, angiogenesis, tumor growth, and malignancy.[60] Other studies have also identified miRNAs secreted from tumor cells that drive tumorigenesis and cell invasiveness upon delivery.[46] Recently, miRNAs within exosomes were found to activate Toll-like receptors within endosomes, leading to oncogenic phenotypes in recipient cells and tumor growth.[62]

Adipocytes may use miRNAs for both local and systemic communication. Large adipocytes were found to transfer miR-16, miR-222, miR-27a, and miR-146b to small adipocytes, which stimulated lipid storage.[63] The miRNA-induced lipid storage may not be contained locally and may serve as a strong metabolic signal resulting from various stimuli. In addition, adipocytes and fat may communicate with inflammatory cells though miRNA transfer.[64] Most interesting, mothers likely use exosomes to shuttle miRNAs to offspring in utero, through release of exosomes into maternal circulation by chronic villi.[65] In addition, embryonic stem cells were found to transfer miRNAs to fibroblasts.[66]

Role in Cardiovascular Disease

Extracellular miRNAs are not inherently insensitive to RNase degradation, as detergents, proteinases, and sonication of extracellular carriers has been reported to render extracellular miRNAs sensitive to digestion.[9,24,32,51,63,67] As such, extracellular carriers likely protect miRNAs from circulating RNases. Extracellular miRNAs, because of their noninvasive accessibility and remarkable stability, hold great potential for disease biomarkers. Altered extracellular profiles have been reported for cardiovascular disease, diabetes mellitus, cancer, nonalcoholic steathepatitis, and multiple other pathophysiologies.[43–45,68,69] At this time, rapid analysis of extracellular miRNA is neither feasible nor available, and purification of the lipid-based carriers is time-consuming. Furthermore, future studies will have to identify altered miRNAs within specific subclasses and demonstrate representative ranges of health and disease in the global profile. Nevertheless, miRNAs likely hold enormous value in diagnostics, early prediction strategies, and pharmacologic assessments.

To date, 2 reports have been published where communication via miRNAs is directly studied in a model of cardiovascular disease.[29,60] Both studies assess the effects of endothelial-secreted microRNAs on the development of atherosclerosis in mice. In the first study, by the Weber laboratory,[29] the authors show that miR-126 is secreted by endothelial

cells via apoptotic bodies, which function as a danger signal for other endothelial cells that obtain antiatherosclerotic properties through the miR-126–mediated repression of RGS16. The second study described the transfer of miR-143 and miR-145 from endothelial cells that were exposed to atheroprotective blood flow to smooth muscle cells.[60] In smooth muscle cells, miR-143 targets ELK1, PHACTR4, and SSH2, and miR-145 targets KLF4, CAMK2d, CFL1, PHACTR4, and SSH2. The suppression of these targets induces a contractile atheroprotective phenotype in the receiving smooth muscle cells.

Extracellular miRNAs and their lipid-based carriers may also be used for novel treatment strategies. By gaining a better understanding and decoding of the unique signals, and comprehending the physiological response, we may aim to enhance or attenuate specific messages for therapeutic approaches. For example, specific miRNAs or inhibitors may be added to specific lipoproteins or extracellular vesicles, as a strategy to alter the extracellular miRNA signature to gain a therapeutic advantage. Reconstituted HDL, recombinant apolipoprotein A-I, and apolipoprotein A-I mimetic peptides each are currently being tested in clinical trials to increase HDL-cholesterol levels and enhance the benefits of HDL.[70] Future studies may look to customize reconstituted particles with unique cassettes of miRNAs for therapeutic gains. Multiple groups have already used lipoproteins and extracellular vesicles to delivery anticancer drugs, modified small interfering RNAs, and enriched miRNAs.[71–73] One could also use natural atheroprotective extracellular vesicles, such as apoptotic bodies from endothelial cells enriched with miR-126 that were previously found to stabilize plaques in atherogenic mice,[29] or extracellular vesicles generated by endothelial cells exposed to atheroprotective stimuli that contain a complex cocktail of beneficial miRNAs, including miR-143 and miR-145.[61]

In 2010, it was reported that breast milk contains exosomes with functional miRNAs.[10] Authors proposed then that lipid-based carriers may transfer functional miRNAs to offspring by crossing the gut lumen.[10,71,74] A subsequent study found that miRNAs found in foods are indeed passed through the gut and enter circulation, as plant miRNAs were observed to be transported to the liver.[71] Nevertheless, both studies support the hypothesis that ingested miRNAs, likely protected by lipid-based carriers, retain functionality and regulate genes in recipient tissues.

Future advances into the role of extracellular miRNAs may include the packaging of miRNAs into lipid-based carriers for dietary supplementation during gestation or nutrition. miRNA intercellular communication likely controls many facets of cardiovascular disease, and novel therapeutic strategies manipulating these networks have tremendous applicability in cardiovascular sciences. Many individuals suffer from ischemic events and morbidity with normal lipid values and statin therapy. Decoding and manipulating the miRNA intercellular message provide a unique opportunity to advance our current approaches.

In summary, analysis of distinct subclasses of miRNA carriers has revealed specific miRNA profiles in health and disease. Basic biology of these carriers has provided many fundamental insights into the mechanisms that govern miRNA export, transport, and delivery. However, much is still to be learned about the selective process of miRNA export and the physiological outcome of each miRNA signaling network. Nevertheless, a novel field of study has emerged that has great potential for the detection, prevention, and treatment of cardiovascular disease.

Open Questions

Functional Relevance In Vivo

Although many in vitro studies have provided fundamental insights into the machinery and mechanisms that govern the export, transport, and delivery of miRNAs from cell to cell, little is understood about the exact nature of extracellular miRNAs in health and disease in vivo. One of the most pressing outstanding questions in the field concerns the necessary payload or concentration of miRNAs that is needed to alter cellular physiology or phenotype. Many of the lipid-based carriers only transport a small number of specific miRNAs in relatively low abundance. In vitro studies have clearly shown that carriers can transfer enough miRNAs to repress gene expression and alter phenotype; however, it remains to be determined what the actual physiological flow of information requires in vivo. Extracellular vesicles collected from stimulated endothelial cells were found to reduce atherosclerosis in *Apoe*$^{-/-}$ mice, which confirmed an earlier study that also demonstrated the potential of miRNA intercellular communication in vivo.[24,61] A more biochemical question relates to the cellular usage of transferred miRNAs. Currently, it is not known whether cells have differential sensitivities or utilization of extracellular miRNAs compared with endogenous miRNAs transcribed and processed within the cell. To our knowledge, studies have yet to demonstrate that extracellular miRNAs are indeed loaded into cellular AGO2–miRNP complexes. Furthermore, it is unknown whether extracellular miRNAs regulate miRNA machinery in the recipient cell through targeting miRNA biogenesis pathways, or whether delivered miRNAs alter recipient cell miRNA transcription or stability.

Selective miRNA Export

Maybe the most important facet of intercellular communication is the selective export of specific miRNAs from the cell. Current evidence suggests that the export profile is not representative of the parent cell and is distinct in abundance and content, although very little is understood about the selection and export process. Multiple studies have found that miRNP complexes associate with multivesicular bodies within endosomes, likely in part of a secretory or turnover process.[75,76] Less is understood regarding how selective miRNAs are partitioned and released in outward budding MPs. Precursor miRNA hairpins have been found within MPs,[77] but, in most cases, it appears that extracellular miRNAs are single-stranded.[26] The current model of miRNA biogenesis suggests that single-stranded extracellular miRNAs must have been completely processed, unwound, and likely loaded into AGO2–miRNP complexes, only to escape during selection or exportation. Because not all extracellular miRNAs are bound to AGO2, it is currently unknown whether AGO2 is necessary for export, or whether miRNAs break free from AGO2 after cellular export. Because AGO2 is not present on lipoproteins, it is very likely that miRNAs can be secreted from cells independent of miRNP complexes.[26] The biogenesis, delivery mechanisms, and sources for each circulating carrier is likely different.

Most importantly, it is unknown whether AGO2–miRNA or other ribonucleoprotein complexes contain lipids. Likewise, it is unknown whether extracellular nonvesicle AGO2–miRNAs are transferred to recipient cells and alter gene expression. It is certainly possible that some extracellular ribonucleoprotein complexes may not act as cellular messengers and not participate in intercellular communication.

Role of Extracellular miRNAs in Disease

Cellular miRNAs have proven to be powerful mediators in response to metabolic cues and cellular stress. Nevertheless, future studies are required to fully understand the role extracellular miRNAs play in adaptive and maladaptive responses to disease. Evidence suggests that there are consistent extracellular miRNAs signatures in health, which may be a novel way to quantify health and differentiate disease. Each miRNA carrier subclass likely has its own distinct miRNA signature in health and disease; however, some miRNAs are certainly shared among the carriers, including miR-223 that was found in both exosomes and HDL.[26] It is likely that vesicles and particles exchange proteins, lipids, and other factors; therefore, it is possible that miRNAs could be exchanged or transferred between similar and disparate carrier types.

Another remaining question to the cell-to-cell transfer hypothesis concerns the physical delivery of miRNAs to the cytoplasm or miRNP complexes of recipient cells. The HDL receptor, scavenger receptor B1, uses a selective core uptake process for HDL-cholesterol ester delivery.[78] The delivery of HDL–miRNAs is dependent on scavenger receptor B1 expression and function; however, it is currently unknown whether selective core uptake is distinct from the miRNA delivery process. Similarly, it is not known whether scavenger receptor B1 mediates the uptake of LDL–miRNAs, or whether the LDL receptor-mediated endocytosis delivers functional LDL–miRNAs to recipient cells. Membrane-derived vesicles have been observed to transfer miRNAs through membrane fusion and endocytosis.[79–82] It is likely that membrane fusion could directly dump miRNAs into the cytosol[79]; however, miRNAs taken up by endocytosis must still cross a phospholipid bilayer to enter the cytosol for target recognition and functionality. As such, it is likely that there are many currently unresolved proteins that act as sensors, carriers, and transporters within the cell membrane of endosomes or lysosomes.

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Disclosures

None.

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