REVIEW

Small but mighty: microRNAs as novel signalling molecules in cancer

Camille A. Mathey-Andrews[1], Minh T. N. Le[2,3]

[1]*Harvard Medical School, Boston, MA 02115, USA*
[2]*Program in Cellular and Molecular Medicine, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA*
[3]*Department of Biomedical Sciences, City University of Hong Kong, Kowloon Tong, Hong Kong*

Correspondence: Minh T. N. Le
E-mail: mle.bms@cityu.edu.hk
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MicroRNAs (miRNAs) are short, noncoding RNAs that silence target messenger RNAs by blocking translation or promoting transcript degradation. While the roles of miRNAs within cells have been extensively characterized, emerging evidence suggests that miRNAs are also transported between cells, providing a novel form of intercellular communication. Circulating miRNAs have been identified, packaged in extracellular vesicles or associated with high-density lipoproteins and Argonaute proteins. Specific extracellular miRNAs have been associated with human cancers. They not only serve as measurable disease biomarkers, but recent findings suggest secreted miRNAs may also mediate crosstalk between cancer cells and other cell types, including those that comprise the prometastatic tumor niche. Previous studies, reviewed here, demonstrate that miRNAs released by cancer cells can be internalized by nearby or distant cells, to modify gene expression and alter the tumor microenvironment. As critical drivers of both oncogenesis and metastasis, miRNAs may be attractive therapeutic targets in a wide range of cancers.

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Introduction

Short, noncoding RNAs known as microRNAs (miRNAs) are endogenous post-transcriptional regulators that play active roles in various physiological processes [1-3]. miRNAs are 19-24 nucleotides long and act to silence gene expression by binding complementary sequences, typically in the 3' untranslated region (UTR) of specific target mRNAs. As a consequence of 3'UTR binding, miRNAs may block translation or promote transcript degradation through the RNA-induced silencing complex (RISC). Previous reports have described miRNAs as key drivers of organismal development, cell differentiation, and homeostasis.

While the roles of miRNAs within cells have been characterized extensively, recent findings indicate that miRNAs can also be transported between cells and thus provide an important mode of intercellular communication [4]. Indeed, substantial evidence suggests that miRNAs secreted by "donor" cells can modify gene expression in nearby or distant "recipient" cells. For example, miRNAs can be transferred from T cells to antigen-presenting cells (APCs) through immune synapses to alter gene expression in APCs [5]. Intercellular transfer of miRNAs may also contribute to maternal-fetal crosstalk [6-8]. miRNAs released by the human placental syncytiotrophoblast enter maternal circulation during pregnancy and maternally derived, immune-related miRNAs have been identified in breast milk. Consistent with a putative role for miRNAs as extracellular signaling molecules, researchers have identified numerous cell-free miRNAs in body fluids such as serum/plasma [9-11], saliva [12], urine [13], semen [14], ascites [15], amniotic fluid [16],

bile [17], and cerebrospinal fluid [18, 19].

As the physiological contributions of miRNAs have been elucidated, it has become increasingly evident that dysregulation of miRNA expression and secretion may promote pathological outcomes [4, 20, 21]. Aberrations in levels of specific, circulating miRNAs have been noted in association with acute myocardial infarction [22-24], diabetes [25], sepsis [26, 27], bipolar disorder [28], and a wide range of cancers [29]. In some cases, circulating miRNA levels appear to correlate with disease status. Remarkable reductions in cancer-associated miRNAs have been demonstrated following chemotherapy and tumor resection, and normalization of plasma miRNAs has been closely correlated with improved prognosis and even remission in some individuals [18, 30].

Conversely, increases in some circulating miRNAs have been associated with poor prognosis and disease progression in leukemia [31], B-cell lymphoma [32], melanoma [33], squamous cell carcinoma [12, 34-37], prostate [38], breast [30, 39-41], ovarian [42], colorectal [43-45], gastric [46, 47], and lung cancers [48-51]. Notably, *in vivo* experiments have demonstrated that overexpression of specific extracellular miRNAs, e.g., miR-9, miR-150, miR-200s, miR-210, miR-105 enhances oncogenesis, angiogenesis and metastasis [52-56]. These data provide compelling evidence that the association between miRNA and disease is complex and that miRNAs are not simply molecular markers. Rather, in their capacity to serve as intercellular signals and potent post-transcriptional modifiers, miRNAs may play influential, functional roles in the origination and progression of disease.

Mode of miRNA secretion

The initial isolation of circulating cell-free miRNA presented an intriguing finding, as it was unclear how secreted RNAs evaded degradation by serum RNAses. However, recent evidence suggests that some miRNAs are secreted from cells in lipid-bilayer enclosed vehicles, known as extracellular vesicles (EVs) [57-59]. EVs are categorized by their mode of biogenesis, with three different types: exosomes, microvesicles, and apoptotic bodies [58]. Exosomes originate through an endolysosomal pathway, and are released from cells when multivesicular bodies (MVBs) fuse with the plasma membrane [60]. They range from 40 to 100 nm in diameter and are enriched with membrane tetraspanins (e.g., CD63, CD9, CD81, CD82). Microvesicles, produced through the direct outward budding of the plasma membrane, are more variable in size (100 nm-1000 nm) and are enriched with membrane phsophatidylserine [61]. Apoptotic bodies also vary in size, reaching up to 4 μm in diameter, but are generated through the fragmentation of dying cells. While techniques for effectively purifying and differentiating between these EVs are still being developed, apoptotic bodies can be distinguished by their contents, namely the DNA and histones of dying cells [58, 62].

Cell-free miRNAs have been identified in all three types of EVs [57, 58, 63]. Apoptotic bodies, for example, have been shown to mediate the transfer of miRNA between damaged endothelial cells and surrounding vascular cells during tissue injury [64]. Hundreds of different miRNAs have been isolated from the total RNA of purified exosomes [63, 64]. Recently, we demonstrated that miRNAs belonging to the miR-200 family are secreted by metastatic breast cancer cells in exosomes and microvesicles [54]. Not only did we observe a robust association between these miRNAs and exosomal marker CD63, we also found that RNAse does not degrade cell-free miRNAs in the absence of a membrane solubilizing detergent, suggesting that secreted miRNAs exist within a lipid bilayer.

miRNA secretion has been most widely studied in the context of exosomes. Although exosomes are derived from multivesicular bodies, they are not necessarily dependent on the endosomal sorting complex required for transport (ESCRT) for their secretion [65]. Instead, mechanisms of exosomal release have been shown to involve both ESCRT-dependent and independent pathways. Exosomal release that does not proceed through ESCRT is highly associated with intracellular ceramide production and several experiments have demonstrated that inhibition of neutral sphingomyelinase 2 (nSMase2), an enzyme that catalyzes ceramide biosynthesis, precludes exosome secretion [66].

In addition to nSMase2, Rab27 GTPases, and their effector proteins, Slp4 and Slac2b, have also been implicated in exosome release [58]. In vivo silencing of Rab27a in breast carcinoma cells injected subcutaneously into mice results in reduced exosome release, as well as decreased lung metastasis, likely due to interruption of exosome-mediated spread of tumorigenic miRNAs and proteins [67].

Apart from EVs, additional mechanisms of miRNA secretion have also been described. Previous work has demonstrated an association between highly stable, exogenous miRNAs and high-density lipoprotein (HDL) [68]. Findings suggest that HDL readily interacts with cell-free miRNAs and delivers them to cells by binding to scavenger receptor class B type I (SR-BI), a class of cell-surface receptors that mediates the selective uptake of HDL and associated miRNAs. Functional miRNAs may also be transferred between cells through gap junctions [69]. Circulating miRNAs have also been identified in complexes with Argonaute proteins outside EVs and HDLs [70-72]. Although these circulating miRNAs are found abundantly in

Table 1. Putative functions of secreted miRNAs in cancer cell signaling

reted miRNA	Donor cell	Target cell	Gene target	Function	Reference
miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, miR-429)	Metastatic breast cancer cells	Poorly-metastatic breast cancer cells	ZEB2, SEC23a	Promote mesenchymal-to-epithelial transition and metastatic colonization of breast cancer cells	[54]
miR-9	Melanoma, lung and colorectal cancer cells	Microvascular endothelial cells	SOCS5	Promotes endothelial cell migration, angiogenesis	[52]
miR-17-92 cluster (miR-92a)	Leukemia cells	HUVECs	ITGA5	Promote endothelial cell migration, angiogenesis	[84]
miR-150	Monocytic leukemia cells	Dermal microvascular endothelial cells	C-MYB	Enhances cell migration	[53]
miR-409	Prostate stromal fibroblasts	Prostate cancer cells	RSU1, STAG2	Promotes epithelial-to-mesenchymal transition	[89]
miR-210	Breast cancer cells	HUVECs	EFNA3	Angiogenesis	[55]
miR-21, miR-29a	Lung carcinoma cells	Macrophages	Toll-like receptors	Induce prometastatic inflammatory response	[83]
miR-223	IL-4-activated macrophages	Breast cancer cells	MEF2C	Enhance breast cancer cell invasion	[85]
miR-135b	Multiple myeloma cells	HUVECs	FIH-1	Promotes angiogenesis	[90]
miR-105	Breast cancer cells	Mammary epithelial cells	ZO-1	Enhances vascular permeability, promote metastasis	[56]
miR-494, miR-542-3p	Adenocarcinoma cells	Lymph node stroma cells and lung fibroblasts	CDH17	Prepare premetastatic niche	[81]
miR-10b	Breast cancer cells	Mammary epithelial cells	HOXD10, KLF4	Promotes breast cancer cell invasiveness	[91]
miR-214	Lewis lung carcinoma cells	Regulatory T cells	PTEN	Immune suppression, cancer immune evasion	[92]
miR-1246	Colorectal cancer cells	HUVECs	PML	Angiogenesis	[45]
miR-221, miR-222	Tamoxifen resistant breast cancer cells	Tamoxifen sensitive breast cancer cells	P27, ERα	Tamoxifen resistance	[51]

HUVECs, Human umbilical vein endothelial cells

human blood, it remains unclear whether vesicle-free circulating miRNAs are targeted to specific cells or are capable of intercellular communication.

miRNAs as biomarkers for cancer

Many miRNAs exhibit tissue-specific patterns of expression [1-3]. Dysregulation of miRNA secretion is associated with diverse pathological conditions, including diabetes [25], liver disease [73], tissue injury [74], and cancer [11]. Accordingly, changes in extracellular miRNA profiles may reflect molecular alterations in the cells from which they are derived, and therefore provide unique pathological signatures that aid in disease diagnosis and inform therapeutic strategy.

Previous findings suggest that cancer cells release specialized EVs, enriched with miRNAs, which are not released by non-neoplastic cells [75]. Indeed, cancer patients often exhibit elevated levels of specific, circulating miRNA species. In some cases, serum miRNA profiles have effectively distinguished cancer patients from healthy controls with high degrees of accuracy [11, 20]. For example, miR-210 [30] and miR-92 [44] are significantly upregulated in the sera of patients with breast cancer and colorectal cancer, respectively, and the ratio of plasma miR-92a/miR-638 levels has been suggested to predict leukemia [31]. Reports from different groups suggest a strong relationship between upregulated levels of circulating miR-200 family miRNAs (miR-200a, miR-200b, miR-200c, miR-141, miR-429) and neoplastic malignancy. For example, serum miR-141 is elevated in prostate cancer patients, while serum miR-200a and miR-200b are elevated in pancreatic cancer patients [38, 76]. miR-200 family members are also elevated in the serum of malignant ovarian cancer [77], metastatic colorectal cancer [78], and metastatic breast cancer patients [79], as well as in the cerebrospinal fluid of patients with brain metastases derived from primary breast and lung cancers [18]. These data suggest that the miR-200 family is a common biomarker for several different types of cancers. Additional listing of extracellular miRNAs as biomarkers for cancer can be found in Weiland *et al* [20].

Specific, circulating miRNA signatures may not only reflect disease, but also disease status. For example, circulating miR-210 levels are significantly reduced in Her2-positive breast cancer patients who are clinically

responsive to trastuzumab treatment, but remain elevated in patients with residual disease [30].

In light of the robust association between dysregulation of extracellular miRNA and cancer, it appears that quantitation of circulating miRNA species can be a powerful tool for diagnosing cancer and monitoring disease progression. Moreover, circulating miRNAs are good biomarkers because they are highly stable and easily detectable, even at low levels, using quantitative RT-PCR technology [20]. Independent studies by Resnick et al. and Zhu *et al*. found that diagnostic tests that measured circulating miRNA in addition to established biomarkers detected cancer with greater sensitivity than either miRNA or biomarker measurement alone [41, 42]. Importantly, a single, rapid procedure is sufficient to accurately measure serum miRNA and describe an individual's miRNA expression profile [20]. Conversely, characterization of standard protein biomarkers, which are often present in low abundance, is more challenging, dependent on antibody availability, susceptible to chance variation, and sometimes misleading in cases of selective isolation and detection.

miRNA profiling would be particularly useful in diagnosing cancers such as pancreatic cancer and glioblastoma, for which current diagnostic procedures are highly invasive and existing methods for monitoring disease progression (e.g., imaging) are often unreliable [18]. Attempts to characterize the blood "miRNome" have supported this idea [80]. In a multicenter study that examined the profiles of 863 different blood-borne miRNAs, Keller and colleagues found that extracellular miRNA is consistently abnormal across several cancers and other diseases [80]. They also found that blood miRNA profiles alone are capable of distinguishing diseased from healthy individuals with an average accuracy of 88.5%. Taken together, these data suggest that incorporating a "liquid biopsy," or assessment of blood-borne nucleic acids, into standard oncological practice may enhance screening and diagnosis for a wide range of malignant conditions.

Roles of extracellular miRNAs in cancer intercellular communication

Not only may cell-free miRNAs be informative biomarkers for cancer, but they may also play active roles in tumor growth and metastasis [52-55, 81, 82]. *In vitro* and *in vivo* experiments have demonstrated that "donor" cancer cells secrete miRNAs packaged in EVs that are internalized by nearby or distant "recipient" cells. Remarkably, these miRNAs are functional and are capable of reprogramming the receiving cells towards a pro-tumorigenic or pro-metastatic phenotype (Table 1). Stromal fibroblasts [81], endothelial cells [52, 53, 56], immune cells [83], other tumor cells [54], and non-neoplastic epithelial cells [82] have all been identified as recipients of these tumorigenic RNA species.

While bioactive miRNAs may be transferred between adjacent cells through gap junctions and immune synapses, direct cell contact is not a necessary condition for miRNA transfer [54, 69, 83]. miRNAs secreted in EVs have been shown to travel stably over long distances in the body [4, 21]. Recent evidence suggests that precursor miRNAs are packaged in exosomes with components of the RISC-loading complex (i.e., Dicer, AGO2, and TRBP) and are processed into mature miRNAs en route to their recipient target cells [82]. This may explain how exosome-bound miRNAs are capable of silencing gene expression immediately following internalization.

EV-mediated miRNA transfer represents a novel and intriguing form of intercellular communication that many cancers appear to have harnessed to promote angiogenesis [52, 53, 55, 84], generate pro-metastatic inflammatory responses [83], confer metastatic potential throughout heterogeneous populations of tumor cells [54], and transform non-neoplastic epithelial cells into tumor-forming cells [82]. Several reports have indicated that miRNAs released by cancer cells act as molecular signals to nearby or distant endothelial cells and stromal fibroblasts, reprogramming them to promote the formation of the tumor microenvironment or premetastatic niche [52, 53, 55, 81, 84]. Rana and colleagues demonstrated that miRNAs in exosomes derived from metastatic rat adenocarcinoma ASML cells are taken up by lymph node stroma cells and lung fibroblasts *in vivo*, enhancing the formation of lung metastases. miRNAs released by metastatic ASML cells silence regulatory RNAs in stroma cells and fibroblasts, resulting in the upregulation of cellular metalloproteases and angiogenesis-promoting genes [81].

Angiogenesis may also be promoted by miR-9, a miRNA that is commonly released in EVs from lung, skin or colorectal tumor cells, and taken up by tumor-associated endothelial cells [52]. Once internalized, miR-9 promotes endothelial cell migration and tumor angiogenesis by activating the JAK-STAT pathway through downregulation of SOCS5. A specific role for miR-9 in pro-angiogenic signaling is supported by data demonstrating that tumor growth and vasculature are reduced, but apoptosis is unaffected, in tumor-bearing mice treated with miR-9 antagomirs. Similar pro-angiogenic effects are mediated by miR-150, which is secreted in EVs from leukemia cells [53]. miR-150 downregulates c-Myb expression and enhances migration of recipient endothelial cells. c-Myb knowdown with siRNA has previously been shown to promote endothelial cell migration, suggesting that

post-transcriptional regulation of c-Myb by miR-150 might contribute to pro-angiogenic changes in endothelial cell phenotype. The contributions of secreted miR-150 to angiogenesis are further supported by evidence demonstrating that changes in cell migration and c-Myb observed in endothelial cells cultured with leukemia EVs are reversed in the presence of miR-150 antagomirs.

Similarly, a putative role for exosomes in mediating intercellular transfer of pro-metastatic miRNAs is supported by findings indicating that loss of nSMase2 activity abrogates the ability of cancer cells to promote angiogenesis and thereby contribute to metastasis [55, 83]. nSmase2 silencing in breast cancer cells has been shown to block exosome production and reduce pro-angiogenic activity in target endothelial cells [55]. This angiogenic potential could be restored, however, through exogenous administration of cancer exosomes, suggesting that EVs and their functional miRNA cargoes influence metastatic capability.

The leading explanation for how miRNAs exert their functions has been attributed to their ability to block translation and silence regulatory RNA. However, Fabbri *et al*. recently found that internalized miRNAs might also serve as ligands for the Toll-like receptor (TLR) family and act as signaling molecules through TLR-mediated cascades [83]. They showed that internalized exosomal miRNA can bind and activate human and murine TLRs located in cellular endosomes, stimulating NF-κB and secretion of cytokines TNF-α and IL-6, and suggested that these inflammatory responses might enhance tumor growth and metastasis. Consistent with this hypothesis, Fabbri *et al*. also found that wild-type mice developed significantly more lung metastases than TLR7-/- mice when injected with metastatic Lewis lung carcinoma cells. Collectively, these results suggest that miRNA activation of TLR-mediated signaling may be an important mechanism by which secreted miRNAs promote the formation of the pro-metastatic niche and mature tumor microenvironment.

Compelling evidence also suggests that miRNA-mediated communication between tumor cells and their local environment is bidirectional [58]. Macrophage-derived miRNAs have been identified in breast cancer cells, where they have been shown to promote tumor cell invasiveness through downregulation of the myocyte enhance factor/β-catenin pathway [85]. Similarly, CD81-positive exosomes secreted by fibroblasts may be internalized by breast cancer cells, enhancing cell motility and formation of protrusions through activation of Wnt-planar cell polarity signaling [86]. While the specific role of fibroblast-derived miRNAs has not been examined, it is plausible that they might play an important part in conferring metastatic properties to tumor cells.

In addition to modulating interactions between tumors and their environment, miRNAs may also be transferred between cancer cells, resulting in the propagation of metastatic properties throughout heterogeneous populations of tumor cells [54]. Recently, we demonstrated that EVs released by highly metastatic mouse breast cancer cells are taken up by poorly metastatic isogenic cells. Furthermore, we found that metastatic cell-derived EVs contain specialized sets of miRNAs, the miR-200 family, that increase metastasis of recipient tumor cells by activating tumor re-epithelialization (mesenchymal-to-epithelial transition, MET) programs. These data, recapitulated in culture and in murine breast cancer models, suggest that by promoting MET in the recipient cells, miR-200 miRNAs enable the colonization of distant metastatic sites. Supporting this hypothesis, we found that antagonizing miR-200 miRNAs greatly reduced the increase in metastasis associated with transfer of EVs between these cells. Importantly, these findings were validated by similar experiments employing human breast cancer cell lines and supported by other studies that showed enrichment of circulating miR-200 miRNAs in patients with metastatic breast cancer.

Future directions and challenges

Accumulating evidence implicates circulating miRNAs as critical drivers of oncogenesis and metastasis, suggesting that they may be effective therapeutic targets in a wide range of cancers. While more research is needed to further elucidate mechanisms of miRNA secretion, uptake, and epigenetic modification, current findings suggest that blocking miRNA transfer between cancer cells and their environment might abrogate tumor growth and metastasis. Recently, we showed that inhibition of miR-200 transfer in breast cancer cells greatly reduced the formation of lung metastases in murine breast cancer models [54]. Similarly, Fabbri *et al*. demonstrated that blocking miRNA-induced intercellular signaling through Toll-like receptors also has anti-cancer effects [83].

Other therapeutic approaches might target the intercellular transfer of miRNAs by preventing the packaging of miRNAs in exosomes or inhibiting their secretion by cancer cells. Suppression of nSMase2, an enzyme implicated in ceramide-dependent exosomal release, has been shown to reduce cancer cell metastasis *in vivo* [83]. Additionally, biogenesis of mature extracellular miRNAs may also be interrupted by preventing loading of the RISC-loading complex (RLC) in exosomes. Future work exploring miRNA processing and delivery systems may illuminate key events in tumorigenesis and metastasis, as well as the kinetics of

miRNA activity in recipient target cells.

Conversely, mechanisms of intercellular miRNA transfer may be reconfigured to deliberately reprogram tumor cells and their microenvironment away from a metastatic tendency. Given their capacity to carry bioactive cargo and their selective uptake by target cells, exosomes may be harnessed as novel drug delivery vehicles [57]. Reports have demonstrated that miRNAs protected by lipid bilayers possess extraordinary stability in circulation. Therefore, it seems likely that exosomes could be used to stably deliver therapeutic siRNAs to target cells, protecting them from degradation by endogenous RNAses [87]. Moreover, exosomes are not as toxic as artificial liposome complexes and can be delivered to a wide range of cell types [88]. Data demonstrating the ability of exosomes to travel long distances and even cross the blood brain barrier further supports their potential utility as vehicles for drug delivery [57].

Future research should also investigate the putative genetic targets of miRNAs released by cancer cells. This work will require continued characterization of circulating, cancer-associated miRNAs and target prediction using, for example, the TargetScan (http://targetscan.org) database [81]. Once gene targets are identified, additional studies should explore what extracellular levels of circulating miRNAs are necessary to transduce intercellular signals, produce gene silencing, and confer pathological phenotypes. Target identification and description of physiologically relevant levels of circulating miRNAs may provide insight into factors involved in establishing the pro-metastatic niche, as well as critical steps of the invasion-metastasis cascade.

Comprehensive characterization of miRNAs and other EV cargoes will enhance our understanding of intercellular signaling in oncogenesis, angiogenesis, and metastasis. As secreted miRNAs continue to be described, additional work should explore the roles of EV proteins, mRNAs, and bioactive lipids in the development of neoplastic malignancy. More sophisticated methods of purifying different classes of EVs and distinguishing them from cellular debris will also be needed to more precisely assess the involvement of apoptotic bodies and microvesicles, in addition to exosomes, in tumorigenic signaling.

Our burgeoning understanding of miRNAs, their roles in controlling gene expression and, most recently, their part in intercellular communication, opens new avenues for understanding oncogenesis and developing new therapeutic strategies. Future advances in cancer biology will require rigorous and innovative approaches for elucidating the protean contributions of these small, but powerful, regulatory RNAs.

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