MicroRNAs: Cobblestones on the Road to Cancer Metastasis

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ABSTRACT: Cancer metastasis is the product of a multistep process during which tumor cells, responding to different intrinsic and extrinsic stimuli, detach from the primary tumor mass, invade the contiguous stroma, migrate over a long distance, and colonize distant organs. Despite the well-established role of protein-coding genes behind such events, emerging evidence suggests how genetic and epigenetic alterations in microRNAs equally contribute to cancer metastasis. In this review, we retrace step-to-step all the most salient phases of the tumor dissemination process, by focusing on the role that specific microRNAs play from the time a cancer cell leaves the primary tumor until it acquires the ability to form secondary tumors at distant sites. We also provide a discussion of relevant conceptual and technological issues that need to be addressed before a microRNA-based therapy might be exploited in the clinical setting for the prevention and cure of the metastatic disease.

KEY WORDS: miRNA, metastasis, EMT, microenvironment, migration, invasion, ECM

ABBREVIATIONS

BM: basement membrane; CAF: cancer-associated fibroblast; ECM: extracellular matrix; EMT: epithelial-mesenchymal transition; LNA: locked nucleic acid; MET: mesenchymal-epithelial transition; miRNA: microRNA; MMP: matrix metalloproteinase; UTR: untranslated region

I. INTRODUCTION

More than 90% of human tumors are carcinomas, malignant neoplasms of epithelial origin. Conversion of a benign tumor into a malignant one begins when morphological changes affect the integrity of the epithelium. Specifically, epithelial cells are converted from highly differentiated, polarized, and organized cells into undifferentiated, isolated, and mesenchymal-like cells with migratory and invasive properties that anticipate their metastasizing proclivity.

Although scantily understood, metastasis is the product of a long series of multiple sequential and interrelated steps by which a cancer cell from a primary tumor begins to invade the contiguous host tissue and enter the systemic circulation through the lymph and the blood vessels, a process referred to as intravasation (Fig. 1). Within the bloodstream, the circulating tumor cell reduces its proliferating rate and survives awaiting the final step of the metastatic process. During this last phase, far away from the primary site, the invading cell exits from the bloodstream (extravasation), probably through mechanisms similar to those that occur during intravasation, adapts, survives, and colonizes the foreign microenvironment in ways that favor the formation of secondary tumors (Fig. 1).

Metastasis represents the trickiest clinical issue in cancer management, making treatment often unsuccessful. Since most deaths from cancer are due to metastasis, a great effort has been made to identify the genes and pathways governing malignant tumor progression, thus uncovering the concept of “metastagenes” as the family of protein-coding genes active in the metastatic process. More recently, it has become evident that in addition to alterations in genes that encode proteins, anomalies in noncoding RNAs similarly contribute to cancer initiation and progression toward metastasis.
this context, particular attention has been paid to a specific class of small noncoding RNAs, termed microRNAs (miRNAs).

miRNAs are endogenous regulatory RNAs of approximately 22 nucleotides in length, shown to play crucial roles in controlling gene expression, mainly at a post-transcriptional level. By hybridizing to at least partially complementary sequences on target mRNAs, miRNAs can induce mRNA degradation or translation inhibition by acting as agents of the RNA interfering machinery. First discovered in Caenorhabditis elegans with the identification of lin-4 and let-7, hundreds of miRNAs have been thereafter found also in vertebrates, including mammalians. Human miRNAs are pervasively transcribed in the nucleus by RNA polymerase II as primary transcripts (pri-miRNAs), hundreds to thousands of nucleotides long and containing both a 5’cap and a poly(A) tail. A stem-loop shape is prerogative of pri-miRNA structure to recruit the ribonuclease III type (RNase III) enzyme Drosha and its cofactor DGCR8, which crop flanks of pri-miRNA releasing an ~ 70 nucleotide stem-loop miRNA precursor (pre-miRNA). The next steps of miRNA biogenesis involve the

FIGURE 1. Functional roles of miRNAs in the metastatic cascade. (1) Epithelial cancer cells undergo EMT and change their morphology from cobblestone epithelial-like to spindle-shaped mesenchymal-like phenotype. (2) During this process, tumor cells undergo actinic cytoskeleton rearrangements and promote the formation of microscopic protrusions to migrate. (3) Role of tumor microenvironment in cancer metastasis: (a) cancer cells induce ECM degradation to invade surrounding stroma; (b) persistent inflammatory conditions encourage metastasis; (c) CAFs are active participants in tumor metastasis. (4) Cancer cells hijack the blood vasculature to promote tumor angiogenesis. (5) Cancer cells enter into the bloodstream and survive the anoikis. (6) Circulating cancer cells stop in specific sites and colonize distant organs. miRNAs that promote or inhibit each step of the metastatic process are indicated in red or green, respectively.

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maturation of the pre-miRNA, which is first exported into the cytoplasm by the nuclear export receptor Exportin-5, then cleaved by a second RNase III, Dicer, into a 19–22 nucleotide long double-stranded RNA.\textsuperscript{5} The miRNA duplex is then unwound into the mature single-stranded form and incorporated into the RNA-induced silencing complex (RISC), which drives the miRNA on to complementary sequences located within the 3’ or 5’ untranslated regions (UTRs) or the open reading frame regions of target mRNAs. Recently, it has been reported that miRNAs may also target specific sequences in gene promoters, thus exerting a function in transcriptional induction.\textsuperscript{6} However, post-transcriptional repression through binding to 3’UTRs of protein-coding genes remains their main mechanism of action.

Globally, due to its intrinsic molecular nature, a single miRNA can coordinate the expression of hundreds of genes, thus regulating a wide range of biological and physiological processes, including development, differentiation, cell growth, and apoptosis. Furthermore, overwhelming evidence suggests that altered expression of miRNAs is a common hallmark of cancer.\textsuperscript{7}

II. CANCER METASTASIS FROM A MICRORNA POINT OF VIEW

The first direct evidence of an involvement of miRNAs in human cancer was reported in 2002 by Calin and colleagues.\textsuperscript{8} The group was characterizing the 13q14 deletion, often the sole chromosomal anomaly associated to human B-cell chronic lymphocytic leukemia. The vain attempt to identify a protein-coding gene causal for leukemia predisposition and residing in the deleted 30-Kb region led the researchers discover two miRNA genes, namely, miR-15a and miR-16-1, located within the locus. Accordingly, both miRNAs are absent or downregulated in most B-cell chronic lymphocytic leukemia patients. Soon after, Cimmino et al. showed the anti-apoptotic factor Bcl-2 as direct target of \textit{miR-15a} and \textit{miR-16-1}, thus providing insight into how the loss or downmodulation of the two miRNAs contribute to tumorigenesis.\textsuperscript{9} Several strands of further evidence highlighted the importance of specific miRNAs as essential actors of cancer initiation events, tumor progression toward malignancy, and ultimately metastasis.\textsuperscript{7}

A. MicroRNAs Orchestrate EMT: Confering a Dynamic Shape

An early key event of the metastatic process is represented by epithelial-mesenchymal transition (EMT). EMT, first discovered as critical to metazoan embryogenesis, organ development, and then essential for the commitment of a tumor epithelial cell toward malignancy, consists of the series of events by which epithelial cells lose most of their epithelial features and gain properties that are typical of mesenchymal cells.\textsuperscript{10} Specifically, during EMT, epithelial cells break contact with their neighbors by losing adherens and tight junctions that keep them stuck together. At that time, they modify their cytoskeleton architecture assuming a spindle-shaped form that allows them to break through the basal membrane of epithelium and easily migrate over a long distance.\textsuperscript{11} The essential hallmarks of EMT include increased expression of mesenchymal markers, such as vimentin and N-cadherin, overexpression of E-cadherin transcriptional repressors (e.g., Snail, Slug, Twist, E47, and ZEB1 and ZEB2), and membrane-to-nuclear localization of β-catenin. Besides genetic mutations, changes in the expression levels of EMT-related genes are often ascribable to sophisticated networks between signaling pathways and miRNAs as well.\textsuperscript{11–13}

In this context, a number of reports identified a role for \textit{miR-200} family members and \textit{miR-205} in modulating EMT upstream of E-cadherin (Fig. 1).\textsuperscript{14–16} Specifically, Gregory et al. demonstrated that induction of EMT in a canine kidney epithelial cell line (MDCK) downregulates \textit{miR-200} family members and \textit{miR-205} expression levels, and that the sole knockdown or restoration of those miRNAs is sufficient to induce or revert EMT in MDCK cells, respectively.\textsuperscript{14} In the same report, the authors identified two transcriptional repressors of E-cadherin, ZEB1 and ZEB2, as direct targets of the miRNAs.\textsuperscript{14} Accordingly, by evaluating the expression of 207 miRNAs in 60 human cell lines, \textit{miR-200} family members were soon after recognized
as highly specific markers to precisely discriminate the epithelial from the mesenchymal cell phenotypes.\textsuperscript{15} On the other hand, our group showed that \textit{miR-205} is downregulated in human prostate cancer cell lines and clinical samples compared to normal counterparts.\textsuperscript{16} Interestingly, tumors from patients with lymph node dissemination of the disease were shown to be characterized by lower \textit{miR-205} expression than node-negative patients, thus indicating an involvement of \textit{miR-205} in prostate cancer metastasis.\textsuperscript{16} Actually, ectopic expression of \textit{miR-205} in metastatic prostate cancer cell lines reversed their EMT phenotype by inducing an upregulation of E-cadherin expression levels, morphological changes, and cytoskeleton rearrangements. Such events were suggested to be driven by the concurrent repression of ZEB2 and protein kinase C epsilon \textit{miR-205}.\textsuperscript{16}

Soon after, two reports identified a feed-forward loop in which ZEB1 and ZEB2 transcriptionally regulate all members of \textit{miR-200} family expression.\textsuperscript{17,18} These findings fascinatingly suggest how miRNAs may work as members of complex networks that, depending on environmental stimuli, stabilize EMT to induce epithelial differentiation of normal cells or promote invasion of cancer cells.\textsuperscript{17}

\section*{B. MicroRNAs in Cell Movement: Tumor Toddlers}

After tumor cells have changed their morphology from cobblestone epithelial-like to spindle-shaped mesenchymal-like, they undergo actin cytoskeleton rearrangements promoting lamellipodia and filopodia formation, which serve them to definitely migrate. Again, \textit{miR-205} has been demonstrated to play a crucial role upstream of such events. In fact, in a prostatic experimental model, our group showed that \textit{miR-205} can hamper tumor cell invasion by downregulating N-chimaerin, a GTPase-activating protein that acts synergistically with Rac1 and Cdc42Hs in inducing simultaneous production of lamellipodia and filopodia.\textsuperscript{16} Accordingly, after \textit{miR-205} ectopic expression, metastatic prostate cancer cells displayed substantial rearrangements of actin cytoskeleton, with tendency toward a predominantly subcortical redistribution of F-actin and loss of stress fibers and filopodia.\textsuperscript{16} Some other miRNAs have been reported to have important regulatory roles in conferring migratory and invasive skills to tumor cells. Ueno et al., in this context, observed that \textit{miR-584} reduces cell motility in renal cell carcinoma cell lines through the direct inhibition of ROCK-1 (Fig. 1).\textsuperscript{19} This latter, activated by RhoA, is known to promote cell migration, probably by regulating endocytic turnover of E-cadherin and promoting actomyosin contractility, which is required by tumor cells to exert locomotory force against their environment.\textsuperscript{20,21} Specifically, in cooperation with Rho GTPases, ROCK-1 mediates a rounded movement that requires elevated levels of actomyosin contractility. Thus, by targeting ROCK-1, \textit{miR-584} may be suggested to act as a crucial regulator of the amoeboid movement.\textsuperscript{19} Besides indirect regulation, miRNAs can directly target Rho GTPases as well. For instance, stable \textit{in vivo} inhibition of \textit{miR-31}, through a miRNA sponge strategy, allows nonaggressive breast cancer cells to metastasize.\textsuperscript{22} Interestingly, this effect does not involve miRNA influence on primary tumor development, but is specifically attributable to \textit{miR-31} ability to impair several steps of metastasis, including local invasion, extravasation, and colonization of distant organs (Fig. 1). Such a pleiotropic effect is achieved via the coordinate repression of a cohort of metastasis-promoting genes, mainly of RhoA, known to efficiently reorganize actin cytoskeleton toward the formation of stress fibers.\textsuperscript{22} Particularly noteworthy is a report by Weinberg's group showing that \textit{miR-10b} is highly expressed in mouse and human metastatic breast cancer cells and positively regulates cell migration and invasion (Fig. 1).\textsuperscript{23} Specifically, the authors demonstrated that the EMT-related transcription factor Twist induced the expression of \textit{miR-10b} by directly binding to its putative promoter. In turn, Twist-induced \textit{miR-10b} production led to translation inhibition of homeobox D10 mRNA, with a downstream increase of the expression of RhoC, a well-characterized promigratory Rho GTPase.\textsuperscript{23} This study emphasizes a concept that this review has not yet tackled: although a widespread downregulation of miRNAs is commonly observed in tumor cells, upregulation of certain miRNAs can also occur during human cancer initiation and progression.
C. MicroRNAs: Control of Microenvironment Forces

Growing evidence shows the existence of dynamic cross talks between epithelial cells and the surrounding stroma, aimed to guarantee tissue homeostasis. Normal stroma, which is composed of different cell types including fibroblasts, myofibroblasts, adipocytes, endothelial cells, pericytes, immune cells, and interstitial extracellular matrix (ECM), operates to prevent tumorigenesis; in contrast, stromal component anomalies not only promote tumor growth, but are also essential to bestowing invasive and metastatic competency. In this regard, interaction between tumor cells and stromal cells or ECM are mediated via membrane junctions and receptors, as well as via secretion of cytokines, chemokines, and growth factors. Compelling evidence provides understandings into how differential expression of miRNAs may influence the secretion of soluble factors, which in turn give rise to heterotypic cell signals aimed at preventing or fostering tumor progression. Moreover, possible mechanisms through which cancer epithelial cells may regulate gene expression within the tumor stroma, and vice versa, are imputable to a direct transfer of miRNAs.

1. MicroRNAs and Invasion through Extracellular Matrix: Tumor Steals Away

Once a tumor is formed, physical constraints, specifically those imposed by ECM, avoid mobile epithelial cells to invade adjacent tissues. Since distant spread requires local invasion, a tumor cell begins to adopt a series of different strategies to overcome such constraints, first by inducing ECM disruption, which is necessary to ultimately invade the underlying mesenchyme. Despite ECM remodeling being tightly coordinated during normal organ development, degradation of ECM structure by excessive production of matrix metalloproteinases (MMPs) is an essential skill required for cancer cells to invade contiguous stroma. MMPs are in fact involved in promoting ECM organization, inflammatory response, tissue remodeling, wound healing, and angiogenesis in normal tissue, but overexpression of these proteinases has a crucial role in sustaining malignancy. In this context, several studies established that MMP production is not only regulated at the transcriptional level, but also post-transcriptionally by miRNAs. Liu et al. illustrated an intriguing network by which miR-222 mediates a reduction of MMP-1 expression levels in oral tongue squamous carcinoma (OTSCC) cell lines, through cis- and transregulatory mechanisms. Specifically, miR-222 both inhibits MMP-1 translation by directly targeting precise sequences located in the 3’-UTR of the gene and decreases MMP-1 promoter activity through direct SOD2 targeting, thus suggesting a role of the miRNA in impairing cell invasion and metastasis in OTSCC cells. Soon afterward, however, miR-222 in association with miR-221 have been shown to enhance cell invasion in non–small cell lung cancer and hepatocellular carcinoma. Specifically, the authors reported that miR-221 and miR-222 are overexpressed in aggressive non–small cell lung cancer and hepatocarcinoma cells as compared to less invasive and/or normal lung and liver cells. Via direct repression of the metalloproteinase inhibitor TIMP3, miR-221 and miR-222 would promote the activation of MMP-3 and MMP-9, actually supporting cell migration and invasion in such cell milieu. This apparent discrepancy concerning miR-222 function indicates that miRNAs may exert opposite roles according to spatial and temporal expression of their mRNA targets and partners (herein, miR-221), thus suggesting that the oncogenic or tumor-suppressive properties of miRNAs, and their potential use as therapeutic targets or tools, should be always contextualized (Fig. 1).

A specialized layer of ECM that separates epithelial cells from the underlying connective tissue is represented by the basement membrane (BM). BM guarantees structural support to cells and influences cellular behavior by acting as a hub to promote and regulate cell-cell and cell-protein interactions. A specialized layer of ECM that separates epithelial cells from the underlying connective tissue is represented by the basement membrane (BM). BM guarantees structural support to cells and influences cellular behavior by acting as a hub to promote and regulate cell-cell and cell-protein interactions. In this context, our group has recently provided insights into how miR-205 pleiotropically hampers prostate cancer metastasis not only by reverting EMT, but also by regulating BM deposition in normal and tumor prostate cells. Specifically, we showed that miR-205 controls the deposition of all components of laminin-332
and its receptor integrin-β4, which form a complex vital for cell-ECM adhesion and interaction in normal human basal cells (Fig.1).33 Because of its capability of bolstering stable anchorage of epithelial prostatic basal cells to basal lamina, miR-205 may represent an essential regulator of normal prostate morphogenesis and tissue integrity. Accordingly, pathological loss of miR-205, as widely observed in prostate cancer, may favor metastasis by creating discontinuities all along BM. Our group showed that replacement of miR-205 in prostate cancer cell lines is able to restore BM deposition and organization of prostate cancer epithelial cells into 3D normal-like acinar structures, probably as a result of miRNA capacity of simultaneously stimulating BM production and decreasing MMP-2 activity in these cells.16,33

2. MicroRNAs and Inflammation: Tumor Recruits Allies

Besides providing structural support to epithelia, BM acts as a fundamental signaling platform. As a result, damages to BM structure or function affect tissue integrity and induce inflammatory responses, which in turn represent an additional important regulator of tumor fate. In fact, whereas inflammation is a useful instrument adopted in response to tissue injuries, persistent inflammatory conditions can increase the risk of cancer and favor metastasis.34,35 The molecular mechanisms underlying inflammation have been the focus of intense investigation, and cell-derived mediators, such as inflammatory cytokines, prostaglandins, NF-κB, reactive oxygen, and nitrogen species, as well as miRNAs are currently recognized as crucial determinants in the inflammatory response.34 In this context, a wealth of published data indicates that miRNAs may influence the establishment of an inflammatory nest reminiscent of the premetastatic niche. For example, besides modulating EMT-related genes, miR-205 ectopic expression in prostate cancer cell lines induces a reduction of the expression levels of IL-6, the accumulation of which has been abundantly shown to contribute to prostate cancer progression.16 On the other hand, by targeting specific sites within IL-24 and IL-32 promoters, miR-205 seems to induce the production of the two cytokines, which are endowed with tumor suppressor activities.36 Similarly, let-7 family members, the expression levels of which are reduced in different types of human cancer, have been shown to directly target several oncogenes, such as HMGAA2 and KRAS, as well as IL-6 (Fig. 1).37–40 In addition to this evidence suggesting miRNAs as regulators of inflammatory process, inflammatory stimuli have been shown to regulate the production of certain miRNAs as well. In this context, an intriguing study by Iliopoulos et al. identified a transient inflammatory signal initiating an epigenetic switch that converts non-transformed breast epithelial cells into cancer cells.40 Through a positive feedback loop involving NF-κB, Lin28, let-7, and IL-6, the malignant status is sustained, thus ultimately promoting metastasis in breast cancer. In particular, the authors described a regulatory circuit in which IL-6 activates STAT3, which in turn transcriptionally activates miR-21 and miR-181b-1 by directly binding to their promoters. Such transient expression of miR-21 and miR181b-1 in human breast cell lines induces the epigenetic switch. Strikingly, miR-21 and miR-181b-1 inhibit PTEN and CYLD tumor suppressors, respectively, leading to increased NF-κB activity, which is actually required to maintain the transformed state.40 In fact, NF-κB directly targets the Lin28 processing factor. In turn, the rapid induction of Lin28 leads to a downregulation of let-7, which represents a key component of the epigenetic switch, since it directly represses IL-6 expression. Overall, STAT3, which is phosphorylated in response to IL-6 during cellular transformation, together with miR-21, miR181b-1, PTEN, and CYLD, is part of a positive feedback loop that underlies an epigenetic switch linking inflammation to tumorigenicity and metastagenicity.40 Consistently, Meng et al. showed that enforced IL-6 production in cholangiocarcinoma cells can epigenetically alter the expression of specific miRNAs.41 In particular, the authors reported that IL-6, the overexpression of which is largely observed in cholangiocarcinoma, modulates the expression of DNA methyltransferase. This leads to a reduction in miR-370 levels, and consequently to the reinstatement of MAP3K8 expression, which is a prometastatic gene recognized as a direct target of
the miRNA.\textsuperscript{41} Furthermore, by using a microarray technology, O’Connell and colleagues showed that after exposure to polyriboinosinic:polyribocytidylic acid or the cytokine IFN-\(\beta\), primary murine macrophages are stimulated to upregulate the expression levels of \textit{miR-155}, which is known to function as an oncogene in several types of human cancer.\textsuperscript{52} Remarkably, \textit{miR-155} has been recently demonstrated to promote the formation of secondary macroscopic tumors in the lung when breast cancer cells overexpressing the miRNA are injected directly into the bloodstream (Fig. 1).\textsuperscript{42} Together, these findings suggest a direct link between miRNAs, inflammation, and cancer. In particular, they define fascinating mechanisms by which miRNAs may modulate the production of inflammatory mediators, which in turn may stimulate tumor cells to produce specific miRNAs, thus ultimately orchestrating metastasis.

3. \textbf{MicroRNAs and Cancer-Associated Fibroblasts: Tumor Corrupts the Enemy}

Cancer-associated fibroblasts (CAFs) are distinctive cell types recognized as constituting part of the carcinoma and increasingly implicated as functional participants in tumor formation and progression.\textsuperscript{54} Similarly to epithelial cancer cells, CAFs may display genetic mutations, but these are only rare events, suggesting that other mechanisms may rule activation of fibroblasts within tumor stroma. In this context, the first evidence of a direct involvement of miRNAs in the dynamic cross talk between tumor and surrounding tissue was given by Aprelikova and colleagues.\textsuperscript{44} The authors identified \textit{miR-31} as the most downregulated miRNAs in CAFs isolated from endometrial cancers as compared with those derived from normal adjacent tissues. Functionally, conditioned medium from CAFs ectopically overexpressing \textit{miR-31} was shown to reduce migration and matrigel invasion of endometrial cancer cell line EC1, implying that \textit{miR-31} may target genes responsible for the secretion of soluble factors implicated in promoting tumor cell sprouting (Fig. 1).\textsuperscript{44} Putative \textit{miR-31} target genes were identified by gene expression profiling of the same CAF samples and the homeobox gene SATB2 was demonstrated to be the direct target through which the miRNA could decrease tumor cell invasion, migration, and scattering. Specifically, SATB2, the expression levels of which are particularly high in endometrial CAFs, is a nuclear matrix–attachment protein involved in chromatin condensation, interaction with other remodeling complexes and regulation of gene transcription. Similarly to osteoblasts, where SATB2 deficiency leads to diminished levels of MMP3 and increased expression of TIMP3, endometrial CAFs express elevated levels of MMP3 and low levels of TIMP3, thus indicating \textit{miR-31} being involved in a pathway engaging SATB2, which is essential for fibroblasts to regulate tissue remodeling and hinder tumor aggressiveness.\textsuperscript{44} Musumeci et al. described a molecular circuitry in which \textit{miR-15} and \textit{miR-16} impair prostate tumor expansion and invasiveness \textit{in vivo} and \textit{in vitro}, by targeting genes, such as \textit{Fgf-2} and its receptor \textit{Fgfr1}, which would otherwise cooperate to promote the tumor-supporting capacity of prostatic CAFs (Fig. 1).\textsuperscript{45} This study suggests that tumor suppressive activity of \textit{miR-15} and \textit{miR-16} is not confined to the cancer cell compartment, but is also shared by the tumor microenvironment, thus providing a proof of concept for the development of one-hit multitarget therapies that concurrently affect both cancer and its microenvironment. Overall, these reports suggest that aberrant miRNA expression in CAFs may lead to anomalies in gene expression, thus contributing to tumor progression. Actually, as essential players of tumor stroma, miRNAs may also act as heterotypic cell–signaling mediators. Specifically, miRNAs produced by one cell type may regulate the secretion of soluble factors, such as cytokines, chemokines, and growth factors, which in turn act on other cell types via canonical ligand-receptor binding, thus influencing tumor outcome. A paradigm of this was given by Yu et al.\textsuperscript{46} The authors showed that although ectopic expression of \textit{miR-17/20} fails to impair cell migration and invasion in invasive MDA-MB-231 breast cancer cell line, cell-conditioned medium from \textit{miR-17/20}–overexpressing noninvasive MCF7 breast cancer cells reduces migration and invasion of MDA-MB-231 cells. These observations suggested that \textit{miR-17/20} regulates cell migration and invasion via heterotypic signals, precisely by regulating cell
secretion, which in turn influences microenvironment cell conduct (Fig. 1). In particular, by reducing cytokeratin 8 secretion, \textit{miR-17/20} suppresses activation of plasminogen, which is an important player in the process of ECM degradation. Furthermore, by targeting precise sequences within interleukin-8 3'UTR, \textit{miR-17/20} impairs invasiveness of breast cancer cells. In this context, carcinoma-derived interleukins have been recently shown to exert a mandatory action on fibroblast activation, which in turn, through secretion of metalloproteases, enhances tumor growth and development of spontaneous metastases. Although the study by Yu et al. does not involve a direct use of fibroblasts, it highlights how epithelial miRNAs, by modulating the secretion of soluble factors, may contribute to reprogram tumor stroma. In fact, tumor stroma may act in ways that favor a more benevolent or malevolent context, thus impairing or promoting tumor progression. A recent report by Bronisz et al. provides a compendium of this concept. The authors identified a Pten--\textit{miR-320--Ets2} tumor-suppressive axis in stromal fibroblasts, which modulates the intercellular communication within tumor microenvironment and is responsible for pathological and molecular events observed in human breast cancer. Specifically, they showed that loss of the tumor suppressor Pten in fibroblasts results in a downmodulation of \textit{miR-320} and upregulation of one of its direct target, ETS2, which is responsible for stimulating secretion of tumor-promoting factors. Accordingly, ectopic expression of \textit{miR-320} in mammary stromal fibroblasts reduces oncogenic secretome and reprograms the transcriptome of neighboring endothelial and epithelial cells in ways that suppress tumor growth and invasiveness. Interestingly, the authors demonstrated that miR-320 secretome signature can distinguish normal from tumor stroma and might be used to robustly predict outcome in breast cancer patients. Because fibroblasts are responsible for the bulk of all the events that occur within stroma, including production and deposition of ECM proteins and tissue renewal and homeostasis, these studies provide insights into how fibroblast-derived and fibroblast-activating miRNAs, working as active participants within tumor stroma, may functionally contribute to cancer progression. D. MicroRNAs and Angiogenesis: Blazing a Trail to a Distant Place

When tumors reach a certain critical diameter (~2 mm), essential nutrients and oxygen become scarce. At this stage, cancer cells hatch out plans for breaking out of the confines of the primary tumor in search of a more suitable place to start forming a new tumor. Generally, when the maximum size has been achieved, cancer cells become hypoxic and spearhead the formation of new vessels, which first provide them with all the metabolites required for continued growth and then serve as tracks for tumor evasion. Hence, the acquisition of a migratory and invasive phenotype represents a necessary but not sufficient condition for escaping. In fact, cancer cells also hijack the blood vasculature to infiltrate it and definitively initiate their treacherous journey toward a distant site.

The mechanism by which new blood vessels are originated through the sprouting of preexisting vessels is referred to as angiogenesis. Angiogenesis is a finely regulated process, orchestrated by a delicate balance between pro- and anti-angiogenic factors. Activators of endothelial cell proliferation and migration are mainly hypoxia-inducible factor 1 alpha (HIF1-\(\alpha\)) and receptor tyrosine kinase ligands, such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF). On the contrary, thrombospondin-1 (Tsp1), which is the first described angiogenic inhibitor, is a negative modulator of endothelial cell growth and motility. Besides changes in the protein levels of such angiogenic activators or inhibitors, a plethora of evidence reveals that miRNAs may also determine the quiescent or angiogenic state of endothelial cells. Fascinatingly, Dews and colleagues demonstrated that Myc-induced \textit{miR-17–92} cluster production endows cancer cells with a tendency to form large and well-perfused tumors. Because fibroblasts are responsible for the bulk of all the events that occur within stroma, including production and deposition of ECM proteins and tissue renewal and homeostasis, these studies provide insights into how fibroblast-derived and fibroblast-activating miRNAs, working as active participants within tumor stroma, may functionally contribute to cancer progression.
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In vivo after subcutaneous or orthotopic injections in syngenic mice, upmodulation of both Ras and Myc generated neoplasms that were on average three times larger than those arising from Ras-transformed cells. Moreover, histological examination revealed that only tumors simultaneously positive for Myc and Ras were able to produce an extremely robust neovascularization, characterized by numerous large-caliber vessels richly perfused with red blood cells. Surprisingly, no inducers of angiogenesis, such as VEGF and HIF1-α, were differentially modulated in Ras and Myc double-positive tumors, compared to Ras-overexpressing tumors. Indeed, Myc has been demonstrated to decrease Tsp1 levels, mainly by reducing mRNA half-life, thus suggesting a regulation at the post-transcriptional level. In this context, miR-17–92 cluster members, already known to be activated by Myc in human lymphocytes and cooperate with Myc during B-lymphomagenesis, were predicted to target specific sequences within Tsp1. Accordingly, the authors showed that miR-17–92 was actually upregulated in tumors coexpressing both Ras and Myc, and that Myc-induced miR-17–92 was directly responsible for the downregulation of Tsp1. In fact, knockdown of the miRNA cluster in Ras-Myc-overexpressing colonocytes restored the expression of Tsp1 in vitro. In addition, ectopic expression of miR-17–92 in Ras-transformed colonocytes phenocopied all the effects produced by Myc. As a whole, these findings suggest that Myc-induced miR-17–92 production may promote tumor growth and vigorous vascularization both in vitro and in vivo by negatively regulating Tsp1, thus establishing for the first time a role for miRNAs in tumor angiogenesis and providing insights into how miRNA modulation may represent a promising therapeutic strategy for treating tumors characterized by complex genetic alterations (Fig. 1). More recently, Anand et al. demonstrated that the upmodulation of miR-132 in the endothelium of human tumors leads to a downregulation of p120RasGAP, thereby acting as an angiogenic switch during normal development and cancer (Fig. 1). In particular, after exposure to conditioned media from breast and pancreatic tumor cell lines, miR-132 was found to be significantly upregulated in HUVEC normal endothelial cells, which otherwise do not express the miRNA. Furthermore, the authors showed that ectopic expression of miR-132 in HUVEC cells considerably increased cell proliferation and tube-forming capacity, as assessed in vitro in a 3D collagen matrix. On the other hand, intraocular injection of an antisense oligonucleotide targeting miR-132 was demonstrated to reduce postnatal retinal vascular development in mice, thus suggesting that miR-132 may regulate normal angiogenesis in vivo. Remarkably, endothelial expression of miR-132 suppressed the expression of p120RasGAP, which is known to act as a crucial negative regulator of vascular development and remodeling by inhibiting Ras activity on VEGF production. On the contrary, a miRNA-resistant version of p120RasGAP reverted the vascular response induced by miR-132. Accordingly, administration of anti-miR-132 inhibited angiogenesis in wild-type mice but not in mice with an inducible deletion of p120RasGAP. Vessel-targeted nanoparticle delivery of anti-miR-132 restored p120RasGAP expression in the tumor endothelium, suppressed angiogenesis and decreased tumor burden in an orthotopic xenograft mouse model of human breast carcinoma. Overall, these data suggest that miR-132 acts as an angiogenic promoter by directly targeting p120RasGAP in endothelial cells, thus abolishing the resting state of vessels and leading to a Ras-dependent induction of neovascularization. Altogether, these studies suggest that miRNAs may regulate tumor angiogenesis through homotypic and heterotypic signaling. Specifically, although tumor capability of hijacking blood vasculature may ensue from a deregulation of certain miRNAs in cancer cells, modulations of endothelial miRNAs expression levels are also required for endothelial cells to switch from a resting to a proliferative state.

E. MicroRNAs and Anoikis: Surviving the Trip

The process through which epithelial cells normally undergo apoptosis after they lose their contact with neighboring cells and ECM is referred to as anoikis and represents an additional barrier to cancer metastasis. To acquire a definitive malignant phenotype and...
survive the trip through the lymph and the blood circulation, tumor cells have to develop mechanisms to resist anoikis. Although not fully understood, the molecular mechanisms promoting anoikis resistance comprise the stimulation of survival signals that are not dependent on ECM contact and the inhibition of apoptotic pathways. Because multiple pathways dictate how invading tumor cells may survive anoikis, it has been postulated that miRNAs may play important roles during induction of anoikis resistance. In this context, Howe et al. demonstrated that by directly targeting different target genes, miR-200c forestalls the acquisition of a migratory and invasive phenotype as well as anoikis resistance in breast and endometrial cancer cells, thus limiting their ability to survive in the bloodstream. In particular, they showed that although miR-200c did not affect apoptosis when endometrial cancer cells were grown attached to plastic, ectopic expression of the miRNA restored anoikis sensitivity in aggressive breast and endometrial cancer cells (Fig. 1).

54 Specifically, after having screened for epigenetically silenced miRNAs and observed the methylation of a subset of miRNAs in prostate tumor cell lines, the authors analyzed their status in vivo by performing methylation-specific PCR on a set of clinical prostate specimens. Such an analysis revealed a consistent methylation silencing of miR-132 in prostate tumors. Furthermore, by using a public data set, the authors analyzed the expression of the miRNA in relation to various clinical parameters and found that low miR-132 levels correlated with high incidence of metastatic events, lymph node invasion, and recurrence-free survival. In addition, they found a negative correlation between miR-132 expression levels and overall tumor grade and stage, which in turn significantly correlated with miR-132 methylated status. Functionally, ectopic expression of the miRNA in PC-3 prostate cancer cell lines evoked cell death by anoikis and, thereby, impeded cell migration and invasion (Fig. 1). Accordingly, two prosurvival proteins, heparin-binding epidermal growth factor and TALIN2, have been confirmed as direct targets of miR-132 and demonstrated to be the direct effectors of such events.

55 Micr. N. S. and Colonization of Distant Organs: the Conquest of the Foreign Land

Out of many thousands of cancer cells that infiltrate blood circulation, only a few survive the trip and even fewer retain the capability of forming macroscopic secondary tumors in a foreign site. In this context, Png and colleagues identified miR-335 as a robust inhibitor of tumor reinitiation (Fig. 1). In a previous study, the authors discovered a set of miRNAs that suppress breast cancer metastasis to lung and bone, which represent the primary sites of clinical metastasis. Specifically, they showed that the expression of these miRNAs was lost in the majority of primary breast neoplasms from patients who developed metastases. Thereafter, the authors investigated the precise role of some of these miRNAs in the different steps of cancer metastasis both in vitro and in vivo. In particular, miR-126 was found to suppress tumorigenesis and metastasis at least, in part, through the inhibition of cancer cell proliferation. On the contrary, miR-335 and miR-206 were demonstrated to suppress migration, invasion, and metastatic colonization without inhibiting overall tumor growth. Precisely, miR-335 inhibited metastasis by targeting a set of metastasis-related genes, including the transcription
factor SOX4 and the ECM protein Tenascin-C. More recently, the same group reported that multiple mechanisms of silencing impinge on the miR-335 locus in metastatic cells. Precisely, LM2 and BoM2 metastatic derivatives of the MDA-MB-231 breast cancer cell line, with tropism to lung and bone, respectively, were shown to display a loss of copy number at miR-335 locus relative to the parental cell line. Similarly, the authors observed a reduction in miR-335 copy number in clinical breast cancer metastases compared to their matched primary cancers. In addition, hypermethylation of a precise CpG island in the miRNA promoter was observed in the metastatic derivatives of a number of breast cancer cell lines. The data suggest that a selective pressure exists to keep miR-335 silenced during cancer progression, which is consistent with the anti-metastatic properties of the miRNA. However, loss of miR-335 was reported to be an early event in breast cancer evolution. By performing serial dilution injection experiments with wild-type and miR-335–expressing LM2 breast cancer cells, the authors found a strong inhibitory role for the miRNA in the early stages of tumor formation in mice. Precisely, they observed that implantation of 5 × 10^3 or 1 × 10^4 cells into the mouse mammary glands produced the formation of comparable numbers of tumors in the cohort injected with control cells compared to that injected with miR-335–transduced cells. In contrast, on implantation of 1000 cells, miR-335–expressing cells failed to form any tumors. As suggested by the authors, the findings would be consistent with a strongly inhibitory role of miR-335 against tumor formation, which would not depend on proliferation. Rather, in the early stages of cancer progression (i.e., when the tumor is small), external forces may act to contrast malignancy. In this context, cancer cells that develop reduced miR-335 levels through epigenetic or genetic means will have a selective advantage in the primary tumor and during the course of metastatic progression. Similarly, since initial stages of tumor colonization retrace the early steps of primary cancer formation, maintained reduced miR-335 expression may help breast cancer cells lead to end metastatic growth at distant sites (Fig. 1).

III. CONCLUSIONS

In recent years, a variety of miRNAs have been experimentally proven to regulate different steps of the cancer dissemination program and, depending on their target genes, they have been acknowledged to exert either pro- or anti-metastatic functions (Fig. 1). Such evidence sets the rationale for the development of therapeutic interventions for the prevention and cure of metastatic disease based on the modulation of specific miRNAs. Potentially, this may be accomplished by either inhibiting pro-metastatic or by overexpressing anti-metastatic miRNAs. For the first purpose, a number of anti-miRNA oligonucleotides with different chemical modifications have been developed to increase target specificity, stability in body fluids, and pharmacokinetics to allow in vivo applications. An example is represented by locked nucleic acid (LNA)–modified oligomers, which harbor RNA bases with an extra bridge connecting the 2’ oxygen and 4’ carbon. In September 2012, a LNA mixmer (composed of DNA and LNA bases) complementary to miR-122, which is vital for the replication cycle of HCV virus, entered clinical phase II trial to be tested as antiviral agent. Two phase I safety studies had been already completed in healthy volunteers and showed no adverse effects, no dose-limiting toxicities, and less proinflammatory reactions compared to other oligomers in clinical experimentation. Overexpression or replacement of antimetastatic miRNAs can be achieved by using either virus-encoded expression vectors or miRNA synthetic precursors, the latter eventually administered in association with atelocollagen.

Despite the encouraging premises, before a miRNA–based therapeutics may be applied to “metastamirs” (i.e., metastasis-related miRNAs), some conceptual issues must be addressed concerning their specific role on the different steps of dissemination. For example, although the role of miR-200 family members in regulating E-cadherin expression and EMT is well established, their influence on metastatic colonization remains controversial. In fact, overexpression of miR-200s is associated with increased risk of metastasis in breast cancer and promotes metastatic colonization in mouse mod-
phenotypes that seem to be in contrast with the ability of miR-200s to revert EMT and reduce migration and invasion of tumor cells. Korpal et al. showed that this can be explained partly through the direct suppression of Sec23a, which ultimately results in a reduced secretion of Igfbp4 and Tinagl1 metastasis-suppressive proteins. In light of this evidence, miR-200 family members may not be envisaged as purely anti-metastatic miRNAs. Similarly, stable expression of miR-155 in 4T1 breast tumor cells was shown to reduce tumor cell dissemination by preventing EMT. However, when tumor cells were injected directly into the bloodstream, miR-155 remarkably promoted macroscopic tumor formation in the lung. Such apparently paradoxical findings highlighted that the context where a given miRNA is expressed is a critical factor: in mammary fat pads, miR-155 prevents tumor dissemination, whereas in the lung, miR-155 apparently maintains the epithelial phenotype of tumor cells that is critical for macroscopic tumor formation.

More generally, such a biphasic role is to be expected for metastamirs, since it would likely provide the phenotypic and functional plasticity necessary for tumor cells to maximize the potential for escaping the primary tumor and colonizing distant organs. EMT in tumor cells is indeed transient: once a metastatic cell has invaded a new tissue, it needs to reverse its mesenchymal to a more epithelial phenotype to efficiently settle down and grow as a metastasis. The contribution of such process, referred to as mesenchymal-epithelial transition (MET), to cancer progression is still unclear. Several reports support the importance of MET at the metastatic site, since it can be predicted by the pronounced epithelial nature of metastases that are often more epithelial than the original primary tumor. During early steps of metastasis, tumor cells may undergo EMT in response to external cues at the invasive front to gain a more motile and invasive mesenchymal phenotype, by downmodulating, for example, miR-200 family members. It is possible that once the disseminated tumor cells reach a secondary organ, miR-200s may be upregulated to allow tumor cells to regain epithelial properties, thus enhancing their colonization abilities. In this context, genomic and transcriptomic analyses of primary tumors and distant metastases have indicated a high degree of similarity, suggesting that MET of the disseminated tumor cell may not be driven by cell intrinsic factors but may be under the influence of the host microenvironment. Similarly to what happens at the primary sites, where mesenchymal stromal cells can induce EMT in cancer epithelial cells, stroma in the target tissue may favor an MET in disseminated tumor cells. If this hypothesis will be experimentally verified, one could envision anti-metastatic approaches based on the targeting of the stroma instead of, or together with, modulating miRNAs in epithelial cells. This concept suggests how the delivery of therapeutics to the cells of interest is crucial to obtain an efficient antemetastatic effect.

More generally, intratumor administration of miRNA modulators could be used to block or impair initial EMT events, local invasion, and intravasation into the bloodstream. However, already disseminated tumor cells in the blood, in the bone marrow, or undetectable micrometastases would be spared by such an approach. This is problematic, given that cancer patients often harbor large numbers of circulating tumor cells in the blood on initial diagnosis. Effective anti-metastatic therapy should hence impair the survival and colonization capabilities of already disseminated tumor cells and not simply prevent detachment from the primary tumor. To this purpose, systemic approaches should be theoretically preferable. Unfortunately, systemic modulation of a given metastamir may be a double-bladed weapon. First, the anti-metastatic effect might hold true for a specific step of dissemination or tissue and not for others, as previously outlined. Furthermore, systemic miRNA modulation may affect normal cells, thus having potential adverse readouts. In fact, the expression and function of miRNAs is strictly regulated in a tissue-dependent manner: the effect of exogenously delivering a miRNA to cells that normally do not express it or, more generally, of altering the physiological dosage of miRNAs in healthy cells is highly unpredictable. As a consequence, before any miRNA-based therapeutics may be envisaged, deep knowledge on the normal expression and function of the miRNA to be targeted in all human tissues should be obtained. In addition, the toxicities of miRNA modulations in nontargeted cells can be limited by developing approaches to deliver miRNA-
based therapeutics specifically to the cells of interest. To this purpose, the use of delivery systems displaying carrier-defined specificity (as, for example, cell-specific immunoliposomes, which have been already used successfully to deliver small interfering RNAs by target-specific cell-surface receptors) might represent a reliable approach for controlled delivery of miRNA-modulating agents to relevant cell targets. Obviously, knowledge of the spectrum of antigens expressed by tumor cells along the whole dissemination program is necessary to efficaciously impair metastasis.

In this review, we provided comprehensive evidence from the literature of a direct involvement of miRNAs in controlling all individual steps of a cancer dissemination program. Although miRNAs appear as promising therapeutic targets or tools due to their ability to simultaneously regulate entire gene networks, a number of relevant conceptual and technological issues still need to be addressed before miRNA-based approaches might be exploited routinely in the clinics for the prevention and cure of the metastatic disease.

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