Chapter 25

Targeting MicroRNAs to Withstand Cancer Metastasis

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Abstract

MicroRNAs are endogenous, regulatory, noncoding small RNAs shown to play a key role in controlling gene expression, mainly at the posttranscriptional level. Several lines of evidence highlighted the importance of selected microRNAs as essential actors of cancer initiation events, tumor progression towards malignancy, and ultimately metastasis. By acting as either prometastatic or antimetastatic factors, microRNAs may represent novel targets or tools to withstand cancer progression. This chapter summarizes the available strategies to manipulate the expression of metastasis-related microRNAs, either by mimicking or inhibiting them, in cell systems and in vivo models. In addition, we provide a broad overview of conceptual and technological issues that need to be addressed before microRNAs might be exploited in the clinical setting for the prevention and treatment of the metastatic disease.

Key words microRNA, Cancer metastasis, miRNA mimics, miRNA inhibitors, miRNA-based therapeutics

1 Introduction

1.1 Cancer Metastasis

Transformation of a benign tumor into a malignant one begins when morphological changes harm the integrity of the epithelium. Indeed, epithelial cells switch from highly differentiated, polarized, and organized cells into undifferentiated, isolated, and mesenchymal-like cells with migratory and invasive properties that anticipate their metastasizing aptitude [1].

Metastasis is the product of a long series of sequential and interrelated steps by which a cancer cell from a primary tumor acquires ability to invade the contiguous host tissue and enter the systemic circulation through the lymph and the blood vessels (intravasation) [2]. Within the bloodstream, the circulating tumor cell reduces its proliferating rate and survives, waiting for the final step of the metastatic process. In 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,

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readjusts, and colonizes the foreign microenvironment to start the formation of secondary tumors [2].

The principal clinical issue in cancer management is represented by metastatic events, which often make the treatment unsuccessful. In this context, a great effort has been made to identify the genes and pathways governing malignant tumor progression, thus disclosing the concept of "metastagenes" as the family of protein-coding genes active in the metastatic process [1]. Lately, it has become evident that, in addition to alterations in genes that encode proteins, anomalies in noncoding RNAs play a similar role in cancer initiation, progression and tumor metastasis [3]. In this context, particular attention has been paid to a specific class of small noncoding RNAs, named microRNAs (miRNAs).

1.2 MicroRNAs

MiRNAs comprise a large family of single-stranded, endogenous, evolutionary conserved, noncoding RNA molecules of 19–25 nucleotides acting as key posttranscriptional regulators of gene expression [4]. Human miRNAs are processed from precursor molecules transcribed in the nucleus by RNA polymerase II, which contributes to the synthesis of a 5′-capped and 3′-polyadenylated primary miRNA transcript (pri-miRNA) [5]. In the canonical pathway, pri-miRNA processing occurs in two steps (*see* Chapter 1). The first nuclear step begins with the cleavage of pri-miRNA by a protein complex composed of the RNase III enzyme Drosha in association with its cofactor DGCR8, which generates a 70–90 nucleotide long precursor miRNA (pre-miRNA) [5]. Pre-miRNA is then assembled into the Exportin-5/RanGTP complex, which prevents its nuclear degradation and facilitates its export into the cytoplasm [6], where the pre-miRNA is processed by a second RNase III, Dicer, into a 19–22 nucleotide long double-stranded RNA, with two nucleotide-long 3′ overhangs at both ends [5, 7]. The miRNA duplex is then unwound into two single stranded molecules: one strand (the mature miRNA) is incorporated into the multi-proteic RNA-induced silencing complex (RISC), whereas the other strand, referred to as star miRNA (miRNA*), is usually degraded [5, 7]. RISC-associated miRNA, through direct interaction with Argonaute (Ago) proteins, drives the complex onto complementary sequences located within the 3′ or 5′ untranslated regions (UTRs) or the open reading frame regions of target mRNAs [5, 7]. Perfect or nearly perfect base-pairing between the 3′-UTR of a mRNA and the first 2–8 bases at the 5′ end of the miRNA (seed sequence) seems to have a dominant role in the selection of target mRNA by miRNAs [7]. Although posttranscriptional repression remains the principal mechanism of action, it has been recently reported that miRNAs may also target specific sequences in gene promoters, thus exerting a function in transcriptional induction [8].

The exact mechanism of miRNA-mediated gene silencing is still unclear, however it is widely known that, depending on the degree of complementarity with its mRNA target, a miRNA may regulate target-gene expression through two mechanisms. MiRNAs that bind mRNA targets with perfect or nearly perfect complementarity induce their cleavage and subsequent degradation. This is the most common mechanism found in plants [9]. Conversely, metazoan miRNAs usually act by binding to partially complementary regions mostly situated in the 3′-UTR, but also in the 5′-UTR or in the open reading frame, of their target mRNAs [10]. This second mechanism induces a drop in protein levels that is independent of mRNA cleavage. This may actually occurs as a consequence of translation repression or deadenylation-dependent decay of target mRNAs [10]. Since a perfect base paring is required only between the target and the seed sequence of the miRNA to guarantee an efficient silencing, an individual miRNA can potentially regulate several target mRNAs, and each mRNA can be targeted by more than one miRNA [11].

Globally, due to such 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 proliferation, and apoptosis [12, 13]. Furthermore, overwhelming evidence suggests that altered expression of miRNAs is a common hallmark of cancer [14]. Specifically, recent findings indicate that human tumors have deregulated expression of miRNAs and that these molecules may play an active role in regulating the different steps of cancer progression [15]. Based on their expression levels (i.e., up- or down-modulated in tumors and/or metastases) and their target genes, miRNAs can exert oncogenic or tumor-suppressive roles [16]. Overall, these findings made evident the potential of miRNAs as new diagnostic or prognostic biomarkers [17]. In addition, the observation that specific miRNAs are endowed with oncogenic or tumor-suppressive functions pointed to the possibility to use them as novel targets or tools for anticancer therapy [16].

2 Role of MicroRNAs in the Different Steps of Cancer Metastasis

In 2002, Calin and colleagues proved for the first time the straight engagement of miRNAs in human cancer [18]. The researchers were characterizing the 13q14 deletion, often the unique chromosomal anomaly associated to human B-cell chronic lymphocytic leukemia, in the effort to identify a protein-coding gene causal for leukemia predisposition [18]. They discovered that two miRNA genes, namely, *miR-15a* and *miR-16-1*, are located within the locus and both are absent or downregulated in most B-cell chronic lymphocytic leukemia patients [18]. Soon after, Cimmino et al.

made clear that Bcl-2, an anti-apoptotic factor, is a direct target of *miR-15a* and *miR-16-1*, thus clarifying how the loss or down-modulation of the two miRNAs could contribute to tumorigenesis [19]. Collectively, these studies suggested a possible direct involvement of miRNAs in the pathogenesis of cancer (*see* Chapter 24). In particular, abnormal expression of miRNAs results in a deregulation of their target genes, which in turn may have pro-tumor or antitumor properties [16]. However, the specific classification of miRNAs as oncogenes or tumor suppressors can be difficult. Firstly, because a single miRNA can regulate multiple targets; secondly, because the expression pattern of miRNAs can differ among tissues and differentiation states, thus implying that a single miRNA may act either as tumor suppressor or oncogene depending on the cellular context and the presence of its targets [16].

Since a number of miRNAs may control the expression of genes with a clear role in tumor cell invasion, migration, and other steps of the dissemination process, such molecules have been suggested as relevant in metastasis [15]. Here, we focus on a series of recent studies that demonstrate the involvement of miRNAs in cancer metastasis and will explore the advances in the knowledge of how miRNAs can regulate the multistep metastatic process. We also provide evidence concerning the possibility to manipulate metastasis-related miRNA functions. In particular, we summarize the most impactful strategies aimed at either mimicking or inhibiting miRNAs with the final purpose of developing innovative therapeutic approaches for the advanced disease.

2.1 MicroRNAs and Epithelial–Mesenchymal Transition

An early key event of the metastatic process is represented by Epithelial–Mesenchymal Transition (EMT), firstly discovered as critical to facilitate tissue remodeling during embryonic development and then as essential for the commitment of a tumor epithelial cell towards malignancy [20]. EMT consists of a series of events by which epithelial cells lose most of their epithelial features and gain properties that are typical of mesenchymal cells [20]. In particular, epithelial cells break contact with their neighbors, by losing adherens and tight junctions [21]. Moreover, they change 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 [21]. Increased expression of mesenchymal markers, like vimentin and N-cadherin, overexpression of E-cadherin transcriptional repressors (e.g., Snail, Slug, Twist, E47, ZEB1 and ZEB2) and membrane-to-nuclear localization of β-catenin are typical hallmarks of EMT. Besides genetic mutations, changes in the expression levels of EMT-related genes are often ascribable to complicated networks between signaling pathways and miRNAs [21–23].

In this context, *miR-200* family members and *miR-205* have been identified as modulators of EMT upstream of E-cadherin [24–26]. Gregory et al. recognized that induction of EMT in a canine kidney

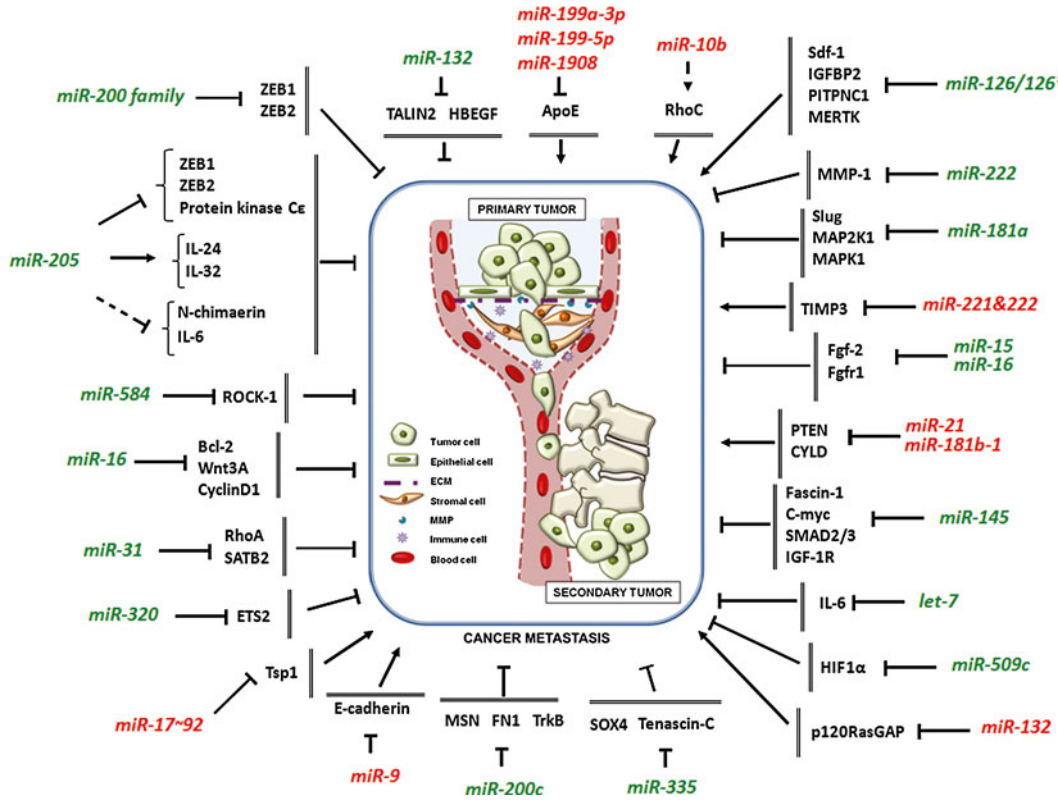

Fig. 1 miRNA-mediated regulation of cancer metastasis. miRNAs that promote or inhibit cancer metastasis are listed in *red* or *green*, respectively. Targeted genes are reported in black. → indicates positive regulation; ⊣ indicates negative regulation. *Solid lines* indicate direct regulation; *dotted lines* indicate indirect regulation

epithelial cell line is able to downregulate *miR-200* family members and *miR-205* expression levels [24]. Moreover, they reported that the sole knockdown or overexpression of such miRNAs is sufficient to induce or revert EMT, respectively [24]. In the same report, the authors identified ZEB1 and ZEB2, two transcriptional repressors of E-cadherin, as direct targets of the miRNAs (Fig. 1) [24]. Accordingly, *miR-200* family members were soon after recognized as highly specific markers to distinguish the epithelial from the mesenchymal cell phenotype [25]. Our group showed that *miR-205* is downregulated in human prostate cancer cell lines and clinical samples compared to normal counterparts [26]. Moreover, we found that tumors from patients with lymph node dissemination of the disease are characterized by lower *miR-205* expression than node-negative patients, thus indicating a possible involvement of *miR-205* in prostate cancer metastasis [26]. Actually, ectopic expression of *miR-205* in metastatic prostate cancer cell lines reversed their EMT phenotype by inducing an up-modulation of E-cadherin expression levels, morphological changes and cytoskeleton rearrangements. The concomitant repression of ZEB2 and protein kinase Cε by *miR-205* has been suggested to drive such events (Fig. 1) [26]. Soon after, two reports identified a

feed-forward loop in which ZEB1 and ZEB2 transcriptionally regulated the expression of all members of *miR-200* family [27, 28]. These fascinating findings 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 [27].

2.2 MicroRNAs and Cell Migration

After tumor cells have changed their morphology from cobblestone epithelial-like to spindle-shaped mesenchymal-like, to definitely migrate they undergo actinic cytoskeleton reorganization promoting lamellipodia and filopodia extrusion [1]. In this context, it has been established that *miR-205* plays a critical role upstream of such events. In fact, our group showed that *miR-205* can hamper prostate cancer cell invasion by downregulating N-chimaerin, a GTPase-activating protein that acts synergistically with Rac1 and Cdc42Hs in inducing simultaneous extension of lamellipodia and filopodia (Fig. 1) [26]. Accordingly, after *miR-205* ectopic expression, metastatic prostate cancer cells show substantial reorganization of actin cytoskeleton, with tendency towards a predominantly sub-cortical redistribution of F-actin and loss of stress fibers and filopodia [26]. In addition, Ueno et al. observed that *miR-584* reduces cell motility in renal carcinoma cell lines through direct inhibition of a crucial regulator of the amoeboid movement, namely, ROCK-1 (Fig. 1) [29]. Similarly, *miR-31* has been shown to impair several steps of breast cancer metastasis, including local invasion, extravasation, and colonization of distant organs, through the coordinate repression of a cohort of metastasis-promoting genes, mainly RhoA (Fig. 1), known to efficiently reorganize actin cytoskeleton towards the formation of stress fibers [30]. An interesting report by Weinberg's group shows that Twist-induced *miR-10b* expression results in the translation inhibition of homeobox D10 mRNA, with a consequential downstream increase of the production of RhoC, a well-characterized pro-migratory Rho GTPase (Fig. 1) [31].

2.3 MicroRNAs and Tumor Microenvironment

Genetic and biological studies show the existence of dynamic cross-talks between epithelial cells and the surrounding stroma, aimed to guarantee tissue homeostasis. Different cell types within normal stroma (e.g., fibroblasts, myofibroblasts, adipocytes, endothelial cells, pericytes, and immune cells) and interstitial extracellular matrix (ECM) operate to prevent tumorigenesis. In contrast, tumor stroma anomalies support not only tumor growth but are also primary to promote invasive and metastatic aptitude [32, 33]. In this regard, membrane junctions and receptors, as well as secretion of cytokines, chemokines, and growth factors, mediate the interaction between tumor cells and stromal cells or the ECM. Compelling evidence offered understandings into how de-regulated expression of miRNAs may alter the secretion of soluble factors, which in turn give rise to heterotypic cell signals aimed at preventing or fostering

tumor development [33]. Moreover, the direct transfer of miRNAs is also imputable as a possible mechanism through which cancer epithelial cells may regulate gene expression within the tumor stroma, and vice versa [1].

2.3.1 MicroRNAs Regulate Extracellular Matrix

When a tumor is formed, physical barriers, specifically the ECM, avoid mobile epithelial cells to escape from the primary site. Since distant spread requires local invasion, a tumor cell begins to adopt a series of different strategies to overcome such constrictions, firstly by inducing ECM remodeling [1]. Loss of ECM structure by overproduction of matrix metalloproteinases (MMPs) is required for cancer cells to infiltrate adjacent tissue [34]. Under physiological conditions, MMPs are the principal actors involved in promoting ECM organization, inflammatory response, tissue remodeling, wound healing, and angiogenesis; however excessive expression of these proteinases has an essential role in sustaining malignancy [34]. Different studies demonstrated that MMP production may be regulated at the posttranscriptional level by miRNAs. Liu et al. illustrated an intriguing network by which *miR-222* mediates, through direct and indirect mechanisms, a reduction of MMP-1 expression levels in oral tongue squamous cell carcinoma cell lines (Fig. 1) [35]. Soon later, it has been shown that miR-221&222, via direct repression of the MMP inhibitor TIMP3 (Fig. 1), promote the activity of MMP-3 and MMP-9, thus promoting cell migration and invasion in non-small-cell lung cancer and hepatocellular carcinoma [36]. This apparent incongruity about *miR-222* role indicates that miRNAs may exert opposite functions depending on spatial and temporal expression of their mRNA targets and partners (herein *miR-221*) [1]. This suggests that the oncogenic or tumor-suppressive properties of miRNAs, and their potential use as therapeutic targets or tools, should be always contextualized [37, 38].

A special organization of ECM is represented by the basement membrane (BM), which separates epithelial cells from underlying connective tissue. BM is not only a structural support to epithelial cells but also influences cell–cell and cell–protein interactions [39]. In this context, our group has recently showed how *miR-205* can pleiotropically hamper prostate cancer metastasis not only by reverting EMT [26], but also by regulating the deposition of all components of BM, in particular laminin-332 and its receptor integrin-β4, which form a complex involved in cell–ECM adhesion and interaction [40]. In particular, we showed that *miR-205* may represent an essential regulator of normal prostate morphogenesis and tissue integrity due to its capability of bolstering stable anchorage of epithelial prostatic basal cells to basal lamina [1]. As a consequence, pathological loss of *miR-205*, as widely observed in prostate cancer, may favor metastasis by creating discontinuities all along BM (Fig. 1) [40]. The replacement of *miR-205* in prostate

cancer cell lines is sufficient to restore BM integrity and organization of cancer epithelial cells into three-dimensional normal-like acinar structures, probably as a result of the miRNA capacity of stimulating BM production and simultaneously decreasing MMP-2 activity [26, 40].

2.3.2 MicroRNAs and Inflammation

Damages to BM structure or function affect tissue integrity and induce inflammatory responses, which in turn represent an additional important regulator of tumor outcome [1]. In fact, whereas inflammation is a useful instrument adopted in response to tissue injuries, lasting inflammatory conditions can amplify the risk of cancer and favor metastasis [41, 42]. The molecular mechanisms supporting inflammation have been the focus of intense investigation. In this context, cell-derived mediators, such as inflammatory cytokines, prostaglandins, NF-κB, reactive oxygen and nitrogen species, as well as miRNAs are currently recognized as principal determinants of inflammatory response [41]. Wealth of published data indicates that miRNAs may influence the establishment of an inflammatory nest, reminiscent of the pre-metastatic niche [1]. For example, *let-7* family members, the expression levels of which are decreased in different types of human cancer, have been shown to directly target several oncogenes, including IL-6 (Fig. 1) [43–46]. Indirectly, ectopic reexpression of *miR-205* in prostate cancer cell lines induces a reduction of the expression levels of the same cytokine [26, 47]. Despite that, by targeting specific sites within promoters of oncosuppressor genes IL-24 and IL-32, *miR-205* induces their expression (Fig. 1) [48]. Besides the 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, Iliopoulos et al. described a regulatory pathway in which, during cellular transformation, IL-6 activates STAT3, which in turn transcriptionally activates *miR-21* and *miR-181b-1* by directly binding to their promoters [46]. Prominently, *miR-21* and *miR-181b-1* directly inhibit PTEN and CYLD tumor suppressors (Fig. 1), respectively, leading to increased NF-κB activity, with a rapid up-modulation of Lin28 and a down-regulation of *let-7*, a direct repressor of IL-6 [46]. Together, these pieces of evidence suggest a clear link between miRNAs, inflammation and cancer. In particular, they define attractive mechanisms by which miRNAs can modulate the production of inflammatory mediators, which in turn may stimulate tumor cells to produce specific miRNAs, thus ultimately orchestrating metastasis.

2.3.3 MicroRNAs and Cancer Associated Fibroblasts

A distinctive cell sub-population of fibroblasts recognized as constituting part of the tumor and actually implicated as functional participants in cancer development and progression are cancer associated fibroblasts (CAFs) [32]. Like epithelial cells, activation of fibroblasts towards malignancy may be achieved upon miRNA

modulations rather than genetic mutations [1]. In this context, Aprelikova and colleagues identified *miR-31* as the most repressed miRNA in CAFs isolated from endometrial cancers as compared with fibroblasts derived from normal adjacent tissues [49]. They also showed that conditioned medium from CAFs ectopically overexpressing *miR-31* reduces migration and Matrigel invasion of endometrial cancer cells, due to the miRNA capability of targeting SATB2 (Fig. 1), a gene essential for fibroblasts to induce tissue remodeling and favor tumor aggressiveness [49]. Similarly, by targeting Fgf-2 and its receptor Fgfr1, *miR-15* and *miR-16* suppress the tumor-supporting capacity of prostatic CAFs impairing prostate tumor progression and invasiveness in vitro and in vivo (Fig. 1) [50]. By revealing that tumor suppressive activity of *miR-15* and *miR-16* is not confined to the cancer cell compartment, this study provides a proof-of-concept for the development of one-hit multitarget therapies which concurrently affect both cancer and its microenvironment [50]. As stromal miRNAs represent essential players of tumor, their modulation may alter the secretion of soluble factors, such as cytokines, chemokines and growth factors, which in turn act on tumor epithelial cells and influence cancer outcome [1]. In this regard, Bronisz et al. demonstrated that the loss of the tumor-suppressor PTEN in fibroblasts results in the downregulation of *miR-320* and up-modulation of one of its direct target, ETS2 (Fig. 1), which is involved in stimulating secretion of tumor-promoting factors [51]. Accordingly, ectopic expression of *miR-320* in mammary fibroblasts reduces oncogenic secretome and reprograms the transcriptome of neighboring endothelial and epithelial cells in ways that suppress tumor growth and invasiveness [51]. Interestingly, the authors found that *miR-320* secretome signature can distinguish normal from tumor stroma and might be used to predict outcome in breast cancer patients [51]. Besides these studies providing insights into how alterations in the expression levels of fibroblast-derived miRNAs functionally contribute to establish and sustain a pro-tumorigenic milieu, we recently demonstrated that, once activated, fibroblasts are also responsible for modulations in the expression levels of cancer cell-derived miRNAs [47]. Specifically, we showed that PC-3 prostate cancer cells exposed to the conditioned medium of patient-derived CAFs undergo a substantial down-modulation in *miR-205* expression levels [47]. In particular, this is mainly due to CAF capability of establishing a pro-oxidant circuit. In fact, CAF stimulation can stabilize redox-sensitive hypoxia inducible factor 1 alpha (HIF1α) protein and trigger its transcriptional repressive activity on *miR-205* promoter in PC-3 cells [47]. The miRNA down-modulation induces PC-3 cells to acquire stemness, invasive and metastatic traits [47]. Surprisingly, the sole restoration of *miR-205* in PC-3 cells under CAFs stimulation is sufficient for them to retrieve a less aggressive phenotype in vitro and in vivo [47]. Overall, by affecting the secretion of

pro-inflammatory cytokines, *miR-205* interrupts the pro-oxidant circuit engaged by reactive stroma, thus acting as a brake against prostate cancer progression and metastasis (Fig. 1) [47].

2.4 MicroRNAs and Angiogenesis

When tumor reaches a certain critical diameter (~2 mm), essential nutrients and oxygen become scarce [1]. Thus, cancer cells become hypoxic and spearhead the generation of new vessels, which first provide them with all the metabolites required for continued growth and then serve as tracks for tumor escape [52]. Therefore, the acquisition of a migratory and invasive phenotype represents a necessary but not sufficient requirement to escape. In fact, cancer cells also hijack the blood vasculature to infiltrate it and definitively migrate to a distant site [52]. Angiogenesis is the process by which new blood vessels are originated through the sprouting of preexisting vessels. It is a finely regulated process, orchestrated by a delicate balance between proangiogenic and antiangiogenic factors [53]. Activators of endothelial cell proliferation and migration are mainly HIF1α and receptor tyrosine kinase ligands, such as vascular endothelial growth factor (VEGF), fibroblast growth factors, platelet-derived growth factor and epidermal growth factor [53]. On the other hand, thrombospondin-1 (Tsp1) is a negative modulator of endothelial cell growth and motility [53]. Plethora of evidence reveals that miRNAs may determine the quiescent or angiogenic state of endothelial cells by changes in the protein levels of such angiogenic activators or inhibitors. Dews and colleagues demonstrated that Myc-induced *miR-17~92* cluster production endows cancer cells with a tendency to form large and well-perfused tumors by targeting specific sequences within Tsp1 mRNA (Fig. 1) [54]. More recently, Anand et al. found that the up-modulation of *miR-132* in the endothelium of human tumors leads to a down-regulation of p120RasGAP (Fig. 1), known to act as a negative regulator of vascular development and remodeling by inhibiting Ras activity on VEGF production [55]. Altogether these studies suggest that miRNAs, through homotypic and heterotypic signaling, can regulate tumor angiogenesis. Specifically, tumor capability of hijacking blood vasculature may ensue from a demodulation of certain miRNAs in cancer cells. However, modulations of endothelial miRNAs are also required for endothelial cells to switch from a resting to a proliferative state [1].

2.5 MicroRNAs and Anoikis

Anoikis is the process through which epithelial cells undergo apoptosis after they lose their contact with neighboring cells and ECM [56]. This event represents an additional barrier to cancer metastasis. In fact, to develop a definitive malignant phenotype and survive the trip through the lymph and the blood circulation, tumor cells need to resist anoikis [56]. The molecular mechanisms supporting anoikis resistance are not clearly understood [56]. It has been postulated that miRNAs may play important roles in this regard.

For instance, Howe et al. demonstrated that *miR-200c* limits breast and endometrial cancer cells ability to survive in the bloodstream by directly targeting pro-survival genes (Fig. 1) (e.g., MSN, FN1, and TrkB) typically expressed in cells of mesenchymal or neuronal origin [57]. Similarly, Formosa et al. have recently observed that silencing of *miR-132* by promoter CpG island methylation represents a critical event contributing to prostate cancer progression [58]. In fact, by directly targeting the pro-survival proteins heparin-binding epidermal growth factor (HBEGF) and TALIN2 (Fig. 1), ectopic expression of *miR-132* in PC-3 cells induced cell detachment and evoked cell death by anoikis [58].

2.6 MicroRNAs and Colonization of Distant Organs

Out of many thousands of cancer cells that infiltrate blood circulation, only a few survive the trip and even less retain the ability of forming macroscopic secondary tumors in a foreign site [1]. In this context, Png and colleagues identified *miR-335* as a robust inhibitor of tumor reinitiation [59]. By targeting a set of metastasis-related genes, including the transcription factor SOX4 and the ECM protein Tenascin-C (Fig. 1), *miR-335* has been shown to inhibit metastasis in breast cancer [60]. Specifically, the authors observed that implantation of 5×10^3 or 1×10^4 cells into the mouse mammary glands produce the formation of comparable numbers of tumors in the cohort injected with control cells compared to that injected with *miR-335*-transduced cells [59]. In contrast, upon implantation of 1×10^3 cells, *miR-335*-expressing cells miss to form any tumors [59]. The authors proposed that the findings were consistent with a strongly inhibitory role of *miR-335* against tumor initiation [59]. In fact, since initial stages of tumor colonization retrace the early steps of primary cancer formation, reduced *miR-335* expression may confer a selective advantage for metastatic growth at distant sites [59].

3 Rise and Fall: Strategies to Modulate miRNAs in Cancer

Several studies have shown that miRNAs, besides being involved in tumor development, may represent leading new strategies for the prevention and treatment of cancer metastasis. Given that miRNAs have multiple target genes, this peculiar property may be exploited to simultaneously modulate a number of metastasis-associated pathways, such as invasion, migration and angiogenesis [61].

Prelude to the advancement of miRNA-based therapeutics is the definition analysis of the precise role of the miRNA of interest in tumor development and progression and the optimization of miRNA-modulating agents. With the aim to study the functional effects of miRNAs in vitro, miRNA expression has been successfully modulated in cellular contexts. When a given miRNA is downregulated in tumor cells, it may have an oncosuppressor activity, and its

reexpression should mitigate the expression of target oncogenes. In contrast, when a miRNA is upregulated, it probably has oncogenic properties, and by abolishing its expression it should be possible to increase the level of a number of tumor-suppressor genes [61].

3.1 Approaches for the Inhibition of miRNAs

The intrinsic structure of miRNAs, which are single-stranded RNA molecules, allows to use Watson and Crick base-pairing interactions to design antisense oligonucleotides for their inhibition [61]. Thus, an anti-miRNA oligonucleotide should be an oligomer that, by strongly binding to a given miRNA, antagonizes its interaction with target mRNAs. Over the years, several chemical modifications have been introduced onto anti-miRNA oligonucleotides, either on sugars or on the backbone. Thanks to these modifications, anti-miRNA oligonucleotides are now characterized by a high stability in both intracellular environments and body fluids and resistance to nucleases [61]. Among anti-miRNAs, 2′-*O*-Methyl (2′-*O*-Me) modified oligomers have been demonstrated to efficiently inhibit the expression of miRNAs both in cell-free extracts and in culture systems [62, 63]. Moreover, these oligomers have been further modified by conjugation with cholesterol (the so-called Antagomirs) in order to target liver-specific *miR-122* in vivo upon systemic administration in mice (Table 1) [64]. Antagomirs have been also successfully used as miRNA-based therapeutics to counteract cancer metastasis in vivo. In this context, Weinberg's group showed that an anti-*miR-10b* was able to reduce the expression levels of the miRNA and concomitantly increase the levels of its target Hoxd10 both in vitro and in vivo (Table 1) [31]. Interestingly, systemic delivery of *miR-10b* antagomirs in tumor-bearing mice suppressed breast cancer metastasis (Fig. 1) [31]. Other chemical modifications at the 2′-position of the sugar, such as 2′-*O*-methoxyethyl (2′-*O*-MOE), 2′-fluoro (2′F) or 2′-F/MOE, have been efficiently used as well [61].

Bicyclic nucleic acid analogues bearing RNA bases with an extra bridge connecting the 2′ oxygen and 4′ carbon, named locked nucleic acids (LNAs), now represent the most widely exploited anti-miRNA molecules. By this modification, a ribose moiety is locked in a C3′-endo conformation thus allowing a considerable enhancement of thermal stability of LNA:miRNA duplex. Specifically, an increase from +2 to 8 °C in duplex melting temperature (T_m) per introduced LNA monomer with respect to unmodified oligomer is obtained [65]. In addition, NMR spectroscopy and X-ray crystallography demonstrated that LNA-modified oligonucleotides are RNA mimics that fit seamlessly into an A-type Watson–Crick duplex geometry similar to that of double-stranded RNA duplexes [66, 67]. Recently, *miravirsen*, an LNA-modified oligomer against *miR-122*, which is essential for the replication cycle of HCV virus, entered a clinical phase IIa trial as antiviral agent. Two phase I safety studies had been already

Table 1
Strategies to inhibit or reconstitute miRNA expression for affecting tumorigenesis and cancer metastasis

Strategy	Targeted miRNA	Disease	Route	Model	Effects	Refs
miRNA inhibition						
Antagomirs (2′-*O*-Methyl modified antisense oligomers)	*miR-10b*	Breast cancer	Systemic administration	Breast cancer xenografts	Reduced expression of *miR-10b* and suppression of breast cancer metastasis	[31]
LNA (locked nucleic acid antisense oligomers)	*miR-199a-3p* *miR-199-5p* *miR-1908*	Melanoma	Systemic administration	Melanoma xenografts	Reduced number of lung metastases	[69]
miRNA sponges	*miR-126/miR-126**	Breast cancer	Ex vivo transfection	4T1 cell line	Increased number of lung metastases in vivo	[73]
	miR-9	Breast cancer	Ex vivo transfection	4T1 cell line	Increased number of metastases in vivo	[74]
miRNA reconstitution						
Synthetic miRNA precursor mimics	*miR-16*	Prostate cancer	Systemic administration	Prostate cancer xenografts	Impaired growth of bone metastases	[80]
Expression plasmid vectors	*miR-509c*	Lung adenocarcinoma	Ex vivo transfection	CL1-5 cell line	Reduced tumor angiogenesis, growth and metastasis in vivo	[81]
Retroviral vectors	*miR-126/miR-126**	Breast cancer	Ex vivo transfection	4T1 cell line	Reduced number of lung metastases	[73]
Lentiviral vectors	*miR-181a*	Salivary adenoid cystic carcinoma	Ex vivo transfection	SACC-LM cell line	Reduced tumor growth and number of lung metastases in vivo	[82]
Adeno-associated viral vectors	*miR-145*	Breast cancer	Intratumoral administration	Orthotopic breast tumors	Reduced tumor growth	[83]

completed in healthy volunteers and showed no adverse effects, no dose-limiting toxicities and less pro-inflammatory reactions compared to other oligomers under clinical development [68]. Although the approach has been developed to treat a non-oncologic disease, available results demonstrated that miRNA-based therapies may have a huge potential also for cancer treatment. For example, in the preclinical setting, Pencheva et al. demonstrated that systemic administration of a cocktail of LNAs targeting *miR-199a-3p*, *miR-199-5p*, and *miR-1908* reduces lung colonization of highly metastatic melanoma cells (Table 1) [69]. Specifically, by targeting the antiangiogenic and metastasis-suppressive factor ApoE, these miRNAs act as endogenous promoters of metastatic invasion, angiogenesis, and colonization in melanoma (Fig. 1). To evaluate the effect of the inhibition of *miR-199a-3p*, *miR-199-5p*, and *miR-1908* on melanoma metastasis prevention in vivo, metastatic melanoma cells were injected into the tail vein of mice and, starting from the following day, a low dose (12.5 mg/kg total) of the LNA cocktail was intravenously administrated biweekly for 4 weeks and then weekly for 7 weeks. Combinatorial LNA treatment successfully reduced lung metastasis without causing weight loss (Table 1) [69].

Another strategy based on "tiny LNA," a 7-8mer LNA-modified oligomer, has been used to target at the same time disparate miRNAs belonging to the same family [70]. The approach is based on the strong binding affinity of tiny LNA to the miRNA seed region. Since miRNAs belonging to the same family share the same seed sequence, tiny LNAs have been shown to enable specific and concentration-dependent inhibition of entire miRNA families in cultured cells with concomitant de-repression of all of their targets [70]. In addition to the aforementioned chemical modifications, antisense molecules can get enhanced nuclease resistance through the introduction of morpholino oligomers or substitution of native phosphodiester with phosphorothioate linkages [65].

Alternative strategies to inhibit miRNAs are "miRNA sponges," expression vectors coding for RNAs containing multiple, tandem binding sites of the miRNA of interest [71]. The strategy allows both transient and long-term miRNA inhibition. For example, to stably knockdown *miR-223* levels in hematopoietic stem cells, Gentner et al. used lentiviral vectors that, carrying at least four imperfectly complementary binding sites for the targeted miRNA, mimic their natural mRNA target sequence thus ultimately sequestering *miR-223* [72]. In addition, by generating a 4T1-derived murine mammary cancer cell line stably expressing miRNA sponges targeting both *miR-126* and *miR-126** (Table 1), Zhang et al. demonstrated how the loss of the two miRNAs enhances the ability of these cells to metastasize to the lung (Fig. 1) [73]. Similarly, Ma et al. showed that a miRNA sponge against *miR-9* (Table 1), a negative regulator of E-cadherin, inhibits metastatic capacity of highly malignant breast cancer cells (Fig. 1) [74].

"MiR-mask" is a strategy based on the opposite idea than miRNA sponges [75, 76]. Indeed, miR-mask is a 22 nucleotide-long single-stranded oligonucleotide carrying other chemical modifications, such as 2′-*O*-methylation, fully complementary to the miRNA binding site within the 3′UTR of a given protein-coding RNA. Therefore, a direct interaction between the miRNA of interest and the antisense oligonucleotide is not required, since such an approach is rather based on masking miRNA binding site on target mRNAs [75, 76].

The last approach to modulate miRNA expression exploits drugs able to modify miRNA transcription. In this context, Gumirreddy et al. found diazobenzene and its derivatives as molecules able to specifically suppress the transcription of *miR-21* [77]. It has been also reported that the small molecule enoxacin is able to enhance the production of several miRNAs with tumor suppressor function by binding to the miRNA biosynthesis protein TAR RNA-binding protein 2 [78]. In this case, the modulation of miRNAs is completely nonspecific. Similarly, modulations of key elements of the miRNA biogenesis pathway, such as Drosha or Dicer, can be exploited to simultaneously affect the expression of thousands of miRNAs. To date, information obtained from studies aimed at silencing the expression of Drosha or Dicer is highly controversial. Specifically, in prostate cancer, Chiosea et al. demonstrated a positive correlation between Dicer expression and poor outcome, but opposite data have emerged for other tumor histotypes [79]. Moreover, it is necessary to keep in mind the existence of alternative miRNA biogenesis pathways that are independent from Drosha or Dicer [61].

Overall, the strategies mentioned so far have been successfully used in cellular and animal models to study the function of miRNAs. For this reason, when properly modified to improve their pharmacokinetic and pharmacodynamic properties, they may be potentially transferred to the clinic in the future [61].

3.2 Approaches for the Replacement of miRNAs

Restoration of miRNA levels can be obtained through different types of molecules with the peculiar characteristic to mimic native miRNAs. Generally, with the aim to faithfully reproduce a physiologic context, double-stranded RNA molecules mimicking natural miRNA precursors are used. Synthetic precursors are usually designed to carry chemical modifications allowing the exclusive production of only the mature miRNA of interest. Moreover, since these molecules have the same sequence as the native miRNAs that need to be reexpressed, they preserve their ability to interact with the natural targets and off-target effects are almost negligible [61]. In this context, Takeshita et al. reported that administration of a synthetic precursor mimicking *miR-16*, a tumor-suppressive miRNA shown to be downregulated in prostate cancer and able to repress Bcl-2, Wnt3A, and cyclin D1 (Fig. 1), inhibits the growth of prostate

tumors in the bone (Table 1) [80]. Specifically, in a therapeutic setting, 50 μg of *miR-16* precursor in association with atelocollagen was administered intravenously into mice at 4, 7, and 10 days after prostate tumor initiation in the bones. On day 28, mice treated with a negative control mimic/atelocollagen complex showed the presence of tumors in the thorax, jaws, and legs. In contrast, the growth of prostate tumors in bone was significantly inhibited by *miR-16* restoration (Table 1). Of note, the effect induced by the miRNA appeared to be restricted to prostate cancer cells, as *miR-16*-treated mice showed no evident toxic off-target effects [80].

The use of synthetic precursors is useful to induce only a transient reexpression of the miRNA of interest [61]. By contrast, a possible strategy to stably restore miRNA expression is represented by cloning miRNA genes into expression vector systems, such as plasmids, to be then transfected into cells. Upon positive selection, cells permanently express the miRNA of interest [61]. Exploiting such a strategy, Cha et al. obtained an adenocarcinoma cell line stably expressing *miR-509c* and observed that upregulation of the miRNA dramatically suppresses tumor angiogenesis, growth and metastasis, probably due to *miR-509c* capability of reducing HIF1α levels (Fig. 1), when the *miR-509c*-overexpressing cells were subcutaneously injected into mice (Table 1) [81]. Other strategies to stably restore miRNA expression are based on the use of retroviral, lentiviral or adeno-associated viral vectors. The main difference between these systems is that, whereas DNA from adeno-associated viruses in any case remains episomal, retroviral or lentiviral vectors can be integrated into the host DNA and the integration is completely unpredictable [61]. However, by assuring stable reexpression of miRNAs, they both adapt well to gene therapy.

Employing a retroviral delivery system, Zhang et al. generated luciferase-labelled 4T1 breast cancer cell populations concomitantly expressing *miR-126* and *miR-126** and implanted them into the mammary fat pad of female mice (Table 1) [73]. Although these cells generated tumors with similar size compared to those originated from parental cells, after surgical removal of the primary mass, mice inoculated with *miR-126/126**-expressing cells were characterized by a reduced number of lung metastases (Table 1) [73]. Similarly, to evaluate the ability of *miR-181a* to counteract tumorigenesis and lung metastasis in vivo, He et al. transfected salivary adenoid cystic carcinoma cells with a GFP-*miR-181a* mimic lentivirus to obtain a stable clone that was injected subcutaneously or into the tail vein of nude mice (Table 1) [82]. The authors observed a significant reduction in the growth of tumors originated from *miR-181a*-overexpressing cells compared to those originated from cells transfected with GFP-control mimic lentivirus. Moreover, evaluation of lung metastases revealed a reduced number of metastatic nodules in mice injected with *miR-181a*-overexpressing cells, as a consequence of direct targeting of MAP2K1, MAPK1, and Slug (Fig. 1) [82].

Besides guaranteeing a highly efficient delivery to target cells, the biggest advantage of viral vectors in the context of a miRNA-based therapy to counteract cancer metastasis is represented by the possibility of injecting them directly into the tumor mass, thus avoiding toxicity arising from the use of lipoplexes. In this regard, the therapeutic potential of *miR-145* against breast cancer has been investigated by Kim et al. (Table 1) [83]. The authors designed an adenoviral construct carrying *miR-145* gene, injected it into mice bearing orthotopically implanted breast tumors and observed that administration of *miR-145* suppresses tumor growth by inhibiting multiple tumor survival effectors such as fascin-1, c-myc, SMAD2/3, and IGF-1R (Fig. 1) [83].

4 Concluding Remarks

Since their discovery, miRNAs have been experimentally proven to be involved in the regulation of several steps of cancer dissemination, thus exerting either prometastatic or antimetastatic functions depending on their target genes [84]. Such evidence piqued researches' interest in the development of therapeutic interventions based on the modulation of specific miRNAs for the prevention and treatment of the metastatic disease. Conceptually, this might be attained by either inhibiting or promoting the expression of prometastatic or antimetastatic miRNAs, respectively. The potential clinical use of miRNA modulating molecules shares some conceptual and technical issues of small interfering RNA-based molecular therapy. However, given their peculiar mechanism of action, miRNA modulators have to be considered as a new class of therapeutics [37].

The main matter to be considered when interfering with the expression of miRNAs concerns the unpredictable off-target effects on unintended mRNA targets and immune activation of "danger signals" [37]. This makes imperative the precise identification and validation of target genes and biological function of miRNAs of interest before a miRNA-based therapy could be reliably applied to metastasis-related miRNAs ("metastamirs") [1]. In the last years, gene expression profiling and proteomic analysis associated to loss- and gain-of-function studies in in vitro or in vivo models of human tumors contributed to the identification of biologically relevant targets and pathways regulated by specific miRNAs, thus defining their precise role in the different steps of cancer development and progression [85, 86]. For example, stable restoration of *miR-155* expression in breast tumor cells has been demonstrated to reduce tumor cell dissemination in vivo when cells were injected in mouse mammary fat pads [87]. This was mainly due to the miRNA capability of reverting EMT. Unexpectedly, injection of *miR-155*-overexpressing tumor cells directly into the bloodstream fostered

the formation of macroscopic tumors in the lung [87]. Similarly, though *miR-200* family members are known to suppress EMT by positively regulating E-cadherin expression, their overexpression seems to be associated with a higher risk of metastasis in breast cancer and promote metastatic colonization in mouse models [88]. This discrepancy can be partly explained by the direct inhibition of metastasis-suppressive proteins driven by *miR-155* or *miR-200* family members [87, 88]. More in general, the biphasic role of miRNAs is largely exploited by tumor cells to progressively gain more aggressive traits [1]. In fact, the possibility of swiftly switching on or off gene expression through little miRNA modulations endows cancer cells with the necessary functional plasticity for either escaping the primary tumor or colonizing distant organs. Indeed, EMT is a transient program that in the first instance allows tumor cells to easily intravasate or extravasate; once invaded a new tissue, metastatic cells have to reverse the mesenchymal traits, engaging a process referred to as mesenchymal–epithelial transition (MET), to settle down and start growing as a secondary tumor [1, 89]. Thus, during the first stages of cancer metastasis tumor cells down-modulate, for example, *miR-200s* to acquire a motile and invasive phenotype and resist to anoikis. Conversely, when tumor cells reach a secondary organ, the upregulation of the same miRNAs provides an advantage for the disseminated cells to enhance their colonizing capacity [1].

EMT/MET program induction is suggested to be ruled by extrinsic, rather than intrinsic, stimuli [89]. In this context, at the primary/distant site the influence of the microenvironment may play an important role for engagement of EMT/MET in cancer cells. If this is experimentally proven, combined approaches based on the targeting of stroma together with miRNA modulation in epithelial cells can be envisaged to counteract metastasis. This concept highlights that not only the timing, but also the context where a given miRNA is expressed is crucial for its definition as a purely prometastatic or antimetastatic miRNA. For this reason, the selective delivery of miRNA-based therapeutics to the cells of interest is crucial to obtain an efficient antimetastatic effect. Theoretically, intratumor administration of miRNA modulators could be used to prevent or impair local invasion and intravasation into the bloodstream [89]. However, such an approach may likely not affect, and in some instance even sustain, survival of already disseminated tumor cells in the blood and in the bone marrow as well as of micrometastases, which are often already present at the initial cancer diagnosis [90]. Thus, besides simply preventing detachment from the primary tumor, effective antimetastatic therapy should impair the survival and colonization capabilities of already disseminated tumor cells [1]. To this purpose, once defined the precise role of a given miRNA, systemic approaches should be preferable. In this context, the main hurdle concerns the risk of unintentionally

affecting miRNAs expression in normal cells. Thus, systemic modulation of given metastamirs may have potential adverse impact. In particular, the effects of altering the physiological dosage of miRNAs in healthy cells, or of delivering a miRNA to cells that otherwise do not express it, are unpredictable. As a consequence, a deep knowledge on the normal expression and function of the miRNA to be targeted should be obtained before any miRNA-based therapeutic may be systemically envisaged. Moreover, to limit the toxicities of miRNA modulations in non-targeted cells, development of approaches to deliver miRNA-based therapeutics specifically to the cells of interest is highly desirable [1]. To this purpose, cell-specific immunoliposomes have been already successfully used to deliver small interfering RNAs by target-specific cell-surface receptors [91]. Obviously, a deep knowledge of the specific antigens exclusively or profusely expressed by tumor cells is instrumental to efficaciously impair metastasis. For instance, prostate-specific membrane antigen (PSMA), specifically expressed on prostate epithelial cells and strongly upregulated in prostate cancer, has been already proven to be a suitable surface molecule to drive prostate cancer-specific delivery of small interfering RNAs [92]. Such an approach might be also exploited for controlled delivery of miRNA-modulating agents to relevant cell targets.

Herein, we provided comprehensive evidence from the literature of a direct involvement of miRNAs in controlling all individual steps of the cancer dissemination program. Due to their ability to simultaneously regulate entire gene networks, miRNAs appear as promising therapeutic targets or tools. However, a broad range of relevant conceptual and technological issues have to be solved, and detailed information concerning the pharmacokinetics of miRNA inhibitors and mimics has to be collected, before miRNA-based therapeutics may be exploited in the clinics for the prevention and treatment of the metastatic disease.

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References

1. Profumo V, Gandellini P (2013) MicroRNAs: cobblestones on the road to cancer metastasis. Crit Rev Oncog 18:341–355
2. Chaffer CL, Weinberg RA (2011) A perspective on cancer cell metastasis. Science 331: 1559–1564
3. Esteller M (2011) Non-coding RNAs in human disease. Nat Rev Genet 12:861–874
4. Pasquinelli AE (2012) MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet 13:271–282
5. Winter J, Jung S, Keller S et al (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 11: 228–234

6. Yi R, Qin Y, Macara IG et al (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17: 3011–3016
7. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297
8. Place RF, Li LC, Pookot D et al (2008) MicroRNA-373 induces expression of genes with complementary promoter sequences. Proc Natl Acad Sci U S A 105:1608–1613
9. Reinhart BJ, Weinstein EG, Rhoades MW et al (2002) MicroRNAs in plants. Genes Dev 16: 1616–1626
10. Guo H, Ingolia NT, Weissman JS et al (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466: 835–840
11. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136: 215–233
12. Ambros V (2003) MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. Cell 113:673–676
13. He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5:522–531
14. Iorio MV, Croce CM (2012) MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med 4:143–159
15. Fiorucci G, Chiantore MV, Mangino G et al (2012) Cancer regulator microRNA: potential relevance in diagnosis, prognosis and treatment of cancer. Curr Med Chem 19:461–474
16. Aigner A (2011) MicroRNAs (miRNAs) in cancer invasion and metastasis: therapeutic approaches based on metastasis-related miRNAs. J Mol Med 89:445–457
17. Cho WC (2010) MicroRNAs: potential biomarkers for cancer diagnosis, prognosis and targets therapy. Int J Biochem Cell Biol 42: 1273–1281
18. Calin GA, Dumitru CD, Shimizu M et al (2002) Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 99:15524–15529
19. Cimmino A, Calin GA, Fabbri M et al (2006) miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 102:13944–13949
20. Hay ED (2005) The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Dev Dyn 233: 706–720
21. Acloque H, Thiery JP, Nieto MA (2008) The physiology and pathology of the EMT. Meeting on the epithelial-mesenchymal transition. EMBO Rep 9:322–326
22. Lee JM, Dedhar S, Kalluri R et al (2006) The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 172:973–981
23. Miska EA (2008) MicroRNAs—keeping cells in formation. Nat Cell Biol 10:501–502
24. Gregory PA, Bert AG, Paterson EL et al (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 10: 593–601
25. Park SM, Gaur AB, Lengyel E et al (2009) The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev 22:894–907
26. Gandellini P, Folini M, Longoni N et al (2009) miR-205 exerts tumor-suppressive functions in human prostate through down-regulation of protein kinase Cepsilon. Cancer Res 69: 2287–2295
27. Burk U, Schubert J, Wellner U et al (2008) A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 9:582–589
28. Bracken CP, Gregory PA, Kolesnikoff N et al (2008) A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res 68:7846–7854
29. Ueno K, Hirata H, Shahryari V et al (2011) Tumour suppressor microRNA-584 directly targets oncogene Rock-1 and decreases invasion ability in human clear cell renal cell carcinoma. Br J Cancer 104:308–315
30. Valastyan S, Reinhardt F, Benaich N et al (2009) A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. Cell 137:1032–1046
31. Ma L, Teruya-Feldstein J, Weinberg RA et al (2007) Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 449:682–688
32. Bissell MJ, Hines WC (2011) Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. Nat Med 17:320–329
33. Wentz-Hunter KK, Potashkin JA (2011) The role of miRNAs as key regulators in the neoplastic microenvironment. Mol Biol Int 2011: 839872

34. Bissell MJ, Radisky D (2001) Putting tumours in context. Nat Rev Cancer 1:46–54
35. Liu X, Yu J, Jiang L et al (2009) MicroRNA-222 regulates cell invasion by targeting matrix metalloproteinase 1 (MMP1) and manganese superoxide dismutase 2 (SOD2) in tongue squamous cell carcinoma cell lines. Cancer Genomics Proteomics 6:131–139
36. Garofalo M, Di Leva G, Romano G et al (2009) miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. Cancer Cell 16:498–509
37. Gandellini P, Profumo V, Folini M et al (2011) MicroRNAs as new therapeutic targets and tools in cancer. Expert Opin Ther Targets 15: 265–279
38. Folini M, Gandellini P, Longoni N et al (2010) miR-21: an oncomir on strike in prostate cancer. Mol Cancer 9:12
39. LeBleu VS, Macdonald B, Kalluri R (2007) Structure and function of basement membranes. Exp Biol Med 232:1121–1129
40. Gandellini P, Profumo V, Casamichele A et al (2012) miR-205 regulates basement membrane deposition in human prostate: implications for cancer development. Cell Death Differ 19: 1750–1760
41. Kundu JK, Surh YJ (2012) Emerging avenues linking inflammation and cancer. Free Radic Biol Med 52:2013–2037
42. Trinchieri G (2012) Cancer and inflammation: an old intuition with rapidly evolving new concepts. Annu Rev Immunol 30:677–706
43. Mayr C, Hemann MT, Bartel DP (2007) Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science 315:1576–1579
44. Johnson SM, Grosshans H, Shingara J et al (2005) RAS is regulated by the let-7 microRNA family. Cell 120:635–647
45. Iliopoulos D, Hirsch HA, Struhl K (2009) An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell 139: 693–706
46. Iliopoulos D, Jaeger SA, Hirsch HA et al (2010) STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. Mol Cell 39:493–506
47. Gandellini P, Giannoni E, Casamichele A et al (2013) miR-205 hinders the malignant interplay between prostate cancer cells and associated fibroblasts. Antioxid Redox Signal. 20:1045–1059
48. Majid S, Dar AA, Saini S et al (2010) MicroRNA-205-directed transcriptional activation of tumor suppressor genes in prostate cancer. Cancer 116:5637–5649
49. Aprelikova O, Yu X, Palla J et al (2010) The role of miR-31 and its target gene SATB2 in cancer-associated fibroblasts. Cell Cycle 9: 4387–4398
50. Musumeci M, Coppola V, Addario A et al (2011) Control of tumor and microenvironment cross-talk by miR-15a and miR-16 in prostate cancer. Oncogene 30:4231–4242
51. Bronisz A, Godlewski J, Wallace JA et al (2011) Reprogramming of the tumour microenvironment by stromal PTEN-regulated miR-320. Nat Cell Biol 14:159–167
52. Stacker SA, Achen MG, Jussila L et al (2002) Lymphangiogenesis and cancer metastasis. Nat Rev Cancer 2:573–583
53. Bergers G, Benjamin LE (2003) Tumorigenesis and the angiogenic switch. Nat Rev Cancer 3:401–410
54. Dews M, Fox JL, Hultine S et al (2010) The myc-miR-17~92 axis blunts TGF{beta} signaling and production of multiple TGF{beta}-dependent antiangiogenic factors. Cancer Res 70:8233–8246
55. Anand S, Majeti BK, Acevedo LM et al (2010) MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. Nat Med 16:909–914
56. Rennebeck G, Martelli M, Kyprianou N (2005) Anoikis and survival connections in the tumor microenvironment: is there a role in prostate cancer metastasis? Cancer Res 65:11230–11235
57. Howe EN, Cochrane DR, Richer JK (2011) Targets of miR-200c mediate suppression of cell motility and anoikis resistance. Breast Cancer Res 13:R45
58. Formosa A, Lena AM, Markert EK (2012) DNA methylation silences miR-132 in prostate cancer. Oncogene. doi:10.1038/onc.2012.14
59. Png KJ, Yoshida M, Zhang XH et al (2011) MicroRNA-335 inhibits tumor reinitiation and is silenced through genetic and epigenetic mechanisms in human breast cancer. Genes Dev 25:226–231
60. Tavazoie SF, Alarcón C, Oskarsson T et al (2008) Endogenous human microRNAs that suppress breast cancer metastasis. Nature 451: 147–152
61. Fenderico N, Casamichele A, Profumo V et al (2013) MicroRNA-mediated control of prostate cancer metastasis: implications for the identification of novel biomarkers and therapeutic targets. Curr Med Chem 20:1566–1584

62. Hutvágner G, Simard MJ, Mello CC et al (2004) Sequence-specific inhibition of small RNA function. PLoS Biol 2:E98
63. Meister G, Landthaler M, Dorsett Y et al (2004) Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. RNA 10: 544–550
64. Krützfeldt J, Rajewsky N, Braich R et al (2005) Silencing of microRNAs in vivo with "antagomirs". Nature 438:685–689
65. Stenvang J, Petri A, Lindow M et al (2012) Inhibition of microRNA function by antimiR oligonucleotides. Silence 3:1
66. Petersen M, Bondensgaard K, Wengel J et al (2002) Locked nucleic acid (LNA) recognition of RNA: NMR solution structures of LNA:RNA hybrids. J Am Chem Soc 124:5974–5982
67. Nielsen KE, Rasmussen J, Kumar R et al (2004) NMR studies of fully modified locked nucleic acid (LNA) hybrids: solution structure of an LNA: RNA hybrid and characterization of an LNA:DNA hybrid. Bioconjug Chem 15: 449–457
68. Santaris.com [Home page in the internet]. Available from: http://www.santaris.com
69. Pencheva N, Tran H, Buss C et al (2012) Convergent multi-miRNA targeting of ApoE drives LRP1/LRP8-dependent melanoma metastasis and angiogenesis. Cell 151: 1068–1082
70. Obad S, dos Santos CO, Petri A et al (2011) Silencing of microRNA families by seed-targeting tiny LNAs. Nat Genet 43:371–378
71. Ebert MS, Neilson JR, Sharp PA et al (2007) MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat Methods 4:721–726
72. Gentner B, Schira G, Giustacchini A (2009) Stable knockdown of microRNA in vivo by lentiviral vectors. Nat Methods 6:63–66
73. Zhang Y, Yang P, Sun T et al (2013) miR-126 and miR-126* repress recruitment of mesenchymal stem cells and inflammatory monocytes to inhibit breast cancer metastasis. Nat Cell Biol 15:284–294
74. Ma L, Young J, Prabhala H et al (2010) miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. Nat Cell Biol 12:247–256
75. Choi WY, Giraldez AJ, Schier AF (2007) Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. Science 318:271–274
76. Wang Z (2011) The principles of MiRNA-masking antisense oligonucleotides technology. Methods Mol Biol 676:43–49
77. Gumireddy K, Young DD, Xiong X et al (2008) Small-molecule inhibitors of microrna miR-21 function. Angew Chem Int Ed Engl 47:7482–7484
78. Melo S, Villanueva A, Moutinho C et al (2011) Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing. Proc Natl Acad Sci U S A 108: 4394–4399
79. Chiosea S, Jelezcova E, Chandran U et al (2006) Up-regulation of dicer, a component of the MicroRNA machinery, in prostate adenocarcinoma. Am J Pathol 169:1812–1820
80. Takeshita F, Patrawala L, Osaki M et al (2010) Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes. Mol Ther 18:181–187
81. Cha ST, Chen PS, Johansson G et al (2012) MicroRNA-519c suppresses hypoxia-inducible factor-1alpha expression and tumor angiogenesis. Cancer Res 70:2675–2685
82. He Q, Zhou X, Li S et al (2013) MicroRNA-181a suppresses salivary adenoid cystic carcinoma metastasis by targeting MAPK-Snai2 pathway. Biochim Biophys Acta 1830: 5258–5266
83. Kim SJ, Oh JS, Shin JY et al (2011) Development of microRNA-145 for therapeutic application in breast cancer. J Control Release 155:427–434
84. Hurst DR, Edmonds MD, Welch DR (2009) Metastamir: the field of metastasis-regulatory microRNA is spreading. Cancer Res 69: 7495–7498
85. Creighton CJ, Nagaraja AK, Hanash SM et al (2008) A bioinformatics tool for linking gene expression profiling results with public databases of microRNA target predictions. RNA 14:2290–2296
86. Yang Y, Chaerkady R, Beer MA et al (2009) Identification of miR-21 targets in breast cancer cells using a quantitative proteomic approach. Proteomics 9:1374–1384
87. Xiang X, Zhuang X, Ju S et al (2011) miR-155 promotes macroscopic tumor formation yet inhibits tumor dissemination from mammary fat pads to the lung by preventing EMT. Oncogene 30:3440–3453
88. Korpal M, Ell BJ, Buffa FM et al (2011) Direct targeting of Sec23a by miR-200 s influences cancer cell secretome and promotes metastatic colonization. Nat Med 17:1101–1108

89. Gao D, Vahdat LT, Wong S et al (2012) Microenvironmental regulation of epithelial-mesenchymal transitions in cancer. Cancer Res 72:4883–4889
90. Pantel K, Brakenhoff RH, Brandt B (2008) Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nat Rev Cancer 8:329–340
91. Pirollo KF, Zon G, Rait A et al (2006) Tumor-targeting nanoimmunoliposome complex for short interfering RNA delivery. Hum Gene Ther 17:117–124
92. McNamara JO 2nd, Andrechek ER, Wang Y et al (2006) Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol 24:1005–1015