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Review

# microRNAs as players and signals in the metastatic cascade: Implications for the development of novel anti-metastatic therapies

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ABSTRACT

microRNAs (miRNAs) are small non-coding RNAs that negatively regulate gene expression at the post-transcriptional level. Increasing evidence emerging from human tumor preclinical models clearly indicates that specific miRNAs, collectively termed "metastamirs," play a functional role in different steps of the metastatic cascade, by exerting either pro- or anti-metastatic functions, and behave as signaling mediators to enable tumor cell to colonize a specific organ. miRNAs also actively participate in the proficient interaction of cancer cells with tumor microenvironment, either at the primary or at the metastatic site. Circulating miRNAs, released by multiple cell types, following binding to proteins or encapsulation in extracellular vesicles, play a main role in this cross-talk by acting as transferrable messages. The documented involvement of specific miRNAs in the dissemination process has aroused interest in the development of miRNA-based strategies for the treatment of metastasis. Preclinical research carried out in tumor experimental models, using both miRNA replacement and miRNA inhibitory approaches, is encouraging towards translating miRNA-based strategies into human cancer therapy, based on the observed therapeutic activity in the absence of main toxicity. However, to accelerate their adoption in the clinic, further improvements in terms of efficacy and targeted delivery to the tumor are still necessary.

## 1. The role of microRNAs in cancer

Metastasis is the result of a multistep process during which cancer cells, responding to a variety of intrinsic and extrinsic stimuli, detach from the primary tumor, invade the contiguous stroma, migrate over a long distance, and colonize different organs [1]. In the classical view of cancer progression, tumor cells acquire the metastatic competence through the accumulation of serial genomic alterations. In the last years, it has become instead clear that the likelihood of bringing to completion the metastatic cascade up to the establishment of clinically relevant metastases relies on a proficient crosstalk between tumor cells and the microenvironment of the organ where cancer is initiated as well as that of the metastatic niche [2,3]. In this context, epigenetic modifications induced by a permissive stroma are not less important than genetic alterations to endow tumor cells with the plasticity needed to go through the different steps of the process. Among distortions in the epigenome, aberrant expression or function of microRNAs (miRNA) has been shown to considerably contribute to cancer metastasis [4,5].

miRNAs comprise a large family of single-stranded, endogenous, evolutionary conserved, non-coding RNA molecules acting as key post-transcriptional regulators of gene expression [6]. 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) [7]. Pri-miRNA processing begins with its cleavage by a protein complex composed of the RNase III enzyme Drosha in association with the cofactor DGCR8, which generates a 70–90 nucleotide long precursor miRNA (pre-miRNA). Pre-miRNA is thereafter assembled into the Exportin-5/RanG-TP complex, which facilitates its export into the cytoplasm, where the pre-miRNA is cleaved by the RNase III Dicer into a 19–22 nucleotide long double-stranded RNA, with two nucleotide-long 3′ overhangs at both ends [7]. The duplex is then separated into two single strands: the mature miRNA and the star miRNA (miRNA*), which is often degraded. The mature miRNA, by interacting with argonaute proteins (Ago) within the multi-protein RNA-induced silencing complex (RISC), guides the complex onto complementary sequences present in the 3′ or 5′ untranslated regions (UTR) or coding regions of its target mRNAs [6,8]. According to the degree of complementarity between the miRNA and its

*Abbreviations:* CAF, cancer associated fibroblast; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; EV, extracellular vesicle; miRNA, microRNA; MSC, mesenchymal stem cells; RISC, RNA-induced silencing complex

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target, the recognition can lead to mRNA cleavage, destabilization or to protein translation inhibition (and subsequent mRNA degradation) [8]. Given that a perfect match is necessary only between the targeted mRNA and the miRNA 'seed' sequence (a critical 2–8 nucleotides stretch located at 5′ end of the miRNA), a single miRNA can potentially regulate the expression of several transcripts, and each transcript can be targeted by more than one miRNA [6]. Such intrinsic feature allows miRNAs to play a role as pivotal rulers of a wide range of biological processes, including development, differentiation, metabolism, proliferation, cell-cycle, apoptosis. Deregulated miRNA expression and/or function have been causatively linked to the pathogenesis of several human diseases, including cancer [9]. In fact, depending on their expression levels, cellular context and functions of their targets, miRNAs can act as oncogenes or tumor suppressors [9]. For example, the down-regulation of a miRNA, the function of which is to mitigate the expression of a given oncogene, may result in aberrant over-expression of the target, thus ultimately promoting cancer [9]. In this regard, well-documented evidence suggests that miRNAs can take part in pathways sustaining all the diverse hallmarks of cancer and therefore actively contribute to tumor development and progression [10].

## 2. MiRNAs governing the different steps of the metastatic cascade: the metastamirs

Increasing evidence emerging from preclinical models of different human tumor types clearly indicates that specific miRNAs, collectively termed "metastamirs," play a functional role in different steps of the metastatic cascade (Fig. 1A) [5].

### 2.1. miRNAs regulating epithelial-to-mesenchymal transition, migration and local invasion

An early event in the metastatic cascade is the epithelial-to-mesenchymal transition (EMT), a complex process characterized by the transcriptional reprogramming of a large set of genes triggered by a small cohort of EMT-associated transcription factors –*e.g.* zinc finger proteins SNAI1 and SNAI2, twist-related protein 1 (TWIST1) and zinc finger E-box-binding homeobox 1 and 2 (ZEB1/2)–, loss of cell polarity and contacts, as well as by the gain of migratory and invasive capabilities associated with metastatic competence [11]. Functional loss of E-cadherin, a pluripotent adhesion molecule necessary to connect adjacent epithelial cells, increased expression of mesenchymal markers, such as vimentin and *N*-cadherin, and membrane-to-nuclear localization of β-catenin are EMT hallmarks. Current knowledge indicates that changes in the expression levels of EMT-related genes are strongly influenced by epigenetic modifications, including aberrant expression of specific miRNAs [12].

A large amount of data suggest that several miRNAs, which are generally down-regulated in different tumor types, act as EMT negative regulators by directly targeting specific EMT-associated transcription factors (miR-1, miR-15b, miR-23b, miR-30c, miR-34a, miR-101, miR-124, miR-132, miR-137 miR-138, miR-150, miR-153, miR-183/96/182, miR-200s, miR-203, miR-204, miR-205, miR-300, miR-335) (Fig. 1A). Additional miRNAs were found to down-regulate the Enhancer of zeste homolog 2 (EZH2) – which participates in DNA methylation and transcriptional repression– (miR-15b, miR-138) or the NAD-dependent deacetylase sirtuin-1 (SIRT1) (miR-200s, miR-204). A number of miRNAs also contribute to EMT inhibition by directly controlling oncogenes involved in different signaling pathways supporting EMT induction, such as TGF-β/Smad, WNT/β-catenin, MAPK/ERK, PI3K/AKT and RhoA/ROCK (miR-26b, miR-29, miR-30b, miR-33a, miR-34b, miR-134, miR-138, miR-141, miR-145, miR-148a, miR-193-3/5p, miR-194, miR-612, miR-638, miR-639) (Fig. 1A) [13].

Thus far, a handful of miRNAs was found to promote EMT by different mechanisms, including direct targeting of E-cadherin (miR-23a, miR-361-5p) or E-cadherin inducers, such as FOXO1 (miR-9), activation of PI3K/AKT pathway through PTEN targeting (miR-21, miR-31, miR-92, miR-221/222) and/or WNT/β-catenin pathway by down-regulating the expression of several pathway suppressors (miR-27, miR-197, miR-374a) (Fig. 1A) [13].

Some of the aforementioned miRNAs, as well as additional ones, also proved to negatively regulate migration and/or invasion ability of tumor cells by affecting the organization of actinic cytoskeleton and inhibiting lamellipodia and fillopodia extrusion (miR-1, miR-205), by directly targeting the main regulators of amoeboid movement ROCK1/2 (miR-135a) or by down-regulating genes encoding extracellular matrix (ECM) proteins (miR-29c) (Fig. 1A) [13–15]. In the other hand, the TWIST1-induced miR-10b was found to promote tumor cell migration and invasion through inhibition of homeobox D10 (HOXD10) and consequent increase in the abundance of the pro-migratory GTPase RhoC [16].

### 2.2. miRNAs regulating intravasation and resistance to anoikis

To disseminate to distant sites, invasive cancer cells must enter the circulatory or lymphatic system. The destruction of vascular endothelial barriers is a critical step for cancer cell intravasation. In this context, miR-182 was found to promote intravasation [17], whereas miR-520c/miR-373 induced the opposite effect (Fig. 1A) [18]. To survive the trip through lymph and blood circulation, tumor cells need to resist anoikis, the process through which epithelial cells undergo apoptosis after they lose their contact with neighboring cells and ECM [19]. It was reported that miR-200c and miR-132 limit cancer cell ability to survive in the bloodstream by directly targeting pro-survival genes [20,21]. In addition, miR-296-3p was found to increase the survival of natural killer cell-resistant circulating prostate cancer cells by targeting intercellular adhesion molecule-1 (ICAM-1) [22]. Such results indicate that specific miRNAs exert opposing effects on the same process, suggesting that the final cell outcome may rely on the relative abundance of the miRNAs and their affected targets.

### 2.3. miRNAs regulating extravasation, colonization to distant organs and angiogenesis

Out of many thousands of cancer cells that infiltrate lymph and blood circulation, only a few survive the trip, adhere to endothelial lining and undergo extravasation. Specific miRNAs were found to affect the ability of such cells to form macroscopic secondary tumors in the foreign site. For example, miR-335 was identified as a robust inhibitor of tumor re-initiation by targeting a set of metastasis-related genes, including the transcription factor SOX4 and the ECM protein Tenascin-C (Fig. 1A) [23].

When the tumor reaches a certain critical diameter, essential nutrients and oxygen become scarce. To face the problem, new blood vessels are originated through the sprouting of preexisting vessels. Experimental evidence indicates that miRNAs may determine the quiescent or angiogenic state of endothelial cells by modulating the expression of angiogenic activators or inhibitors. In this context, it was shown that over-expression of miR-21 in prostate cancer cells enhances hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression as a consequence of AKT/ERK activation [24]. Conversely, restoration of miR-146a was found to reduce microvessel density in subcutaneous prostate cancer xenografts through epidermal growth factor receptor (EGFR) down-regulation [25].

## 3. Novel concepts in the field of metastamirs

### 3.1. Role of miRNAs in tumor microenvironment

The role of miRNAs has been extensively investigated in tumor cells to get insight into their participation to the different steps of the metastatic cascade. However, it is now well acknowledged that

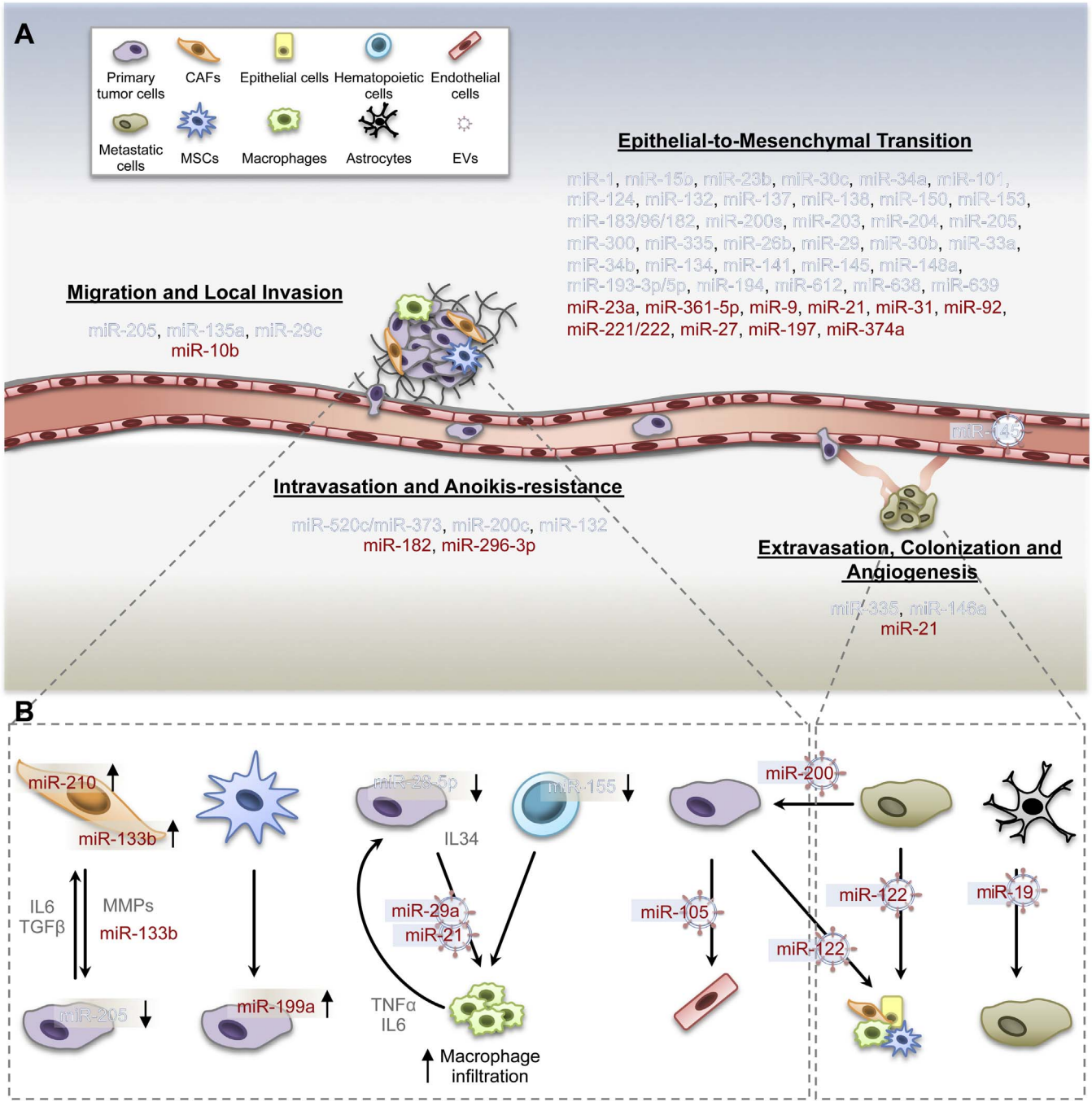


**Fig. 1.** A) Overview of miRNAs involved in the regulation of the different steps of the metastatic cascade. To disseminate to distant organs, tumor cells undergo EMT, detach from the primary site, intravasate into blood vessels and survive in circulation, mainly due to resistance to anoikis. When disseminated tumor cells reach the secondary site, they must extravasate from the bloodstream and colonize the organ. Specific miRNAs may act as positive regulators (highlighted in red) or negative regulators (highlighted in blue) of the process. We refer the reader to the main text ("miRNAs governing the different steps of the metastatic cascade: the metastamirs") for more detailed information on the individual miRNAs. B) Examples of miRNAs involved in the interaction between tumor cells and different types of non-tumor cells, including extracellular miRNAs (either protein-bound or vesicle-associated). We refer the reader to the main text ("Role of miRNAs in tumor microenvironment" and "Extracellular miRNAs as novel signaling molecules") for more detailed information on the individual miRNAs.

metastasis is not a cell-autonomous process. Proficient interaction of cancer cells with tumor microenvironment, intended as the complex assortment of non-tumor cells (including cancer-associated fibroblasts (CAF), endothelial cells, normal epithelial cells, pericytes, mesenchymal stem cells (MSC) and immune-related cells) and the ECM, is a key determinant for the acquisition of a metastatic behavior [26]. In this context, miRNA expression was reported to be modulated in tumor cells in response to different microenvironmental signals and be directly responsible for phenotypic outcomes [27]. For example, in prostate cancer, CAFs have been shown to enhance the metastatic potential of tumor cells through the secretion of metallopreoteinases, which elicit a pro-oxidative and pro-inflammatory cascade culminating in HIF-1α –mediated repression of miR-205 (Fig. 1B) [28–30]. In turn, down-modulation of the miRNA results in derepression of its target genes, such as E-cadherin transcriptional repressors ZEB1/2, ultimately inducing EMT and favoring metastatic dissemination [30,31].

Similarly, it has been shown that bone marrow-derived MSCs can trigger the modulation of a set of miRNAs in breast cancer cells, which, spearheaded by miR-199a, converge on and repress the expression of the transcription factor forkhead-box P2 (Fig. 1B) [32]. This leads to the

acquisition of cancer stem cell traits, enhancement of tumor-initiating capability and fostering of metastatic potential [32].

MiRNA changes occurring in tumor cells may also impinge on the behavior of adjacent cells, rather than on intrinsic cancer cell features. For example, miR-28-5p deficiency was found to increase hepatocellular cancer growth and metastasis in nude mice without altering the biological characteristics of tumor cells (Fig. 1B) [33]. In fact, down-modulation of the miRNA results in increased expression of its target interleukin-34 (IL34). Enhanced secretion of IL34 in turn stimulates macrophages to infiltrate the tumor, where they were shown to favor metastasis [33].

As interaction between cancer cells and their microenvironment is a bidirectional process, changes in miRNAs have been also observed in stromal cells upon stimulation by tumor-derived signals.

For instance, establishment of a reactive stroma (intended as the presence of myofibroblast-like cells similar to those found during wound healing) in tumors is indeed achieved through the activation of resident fibroblasts by cytokines released by cancer cells [34]. In prostate cancer, it was shown that stimulation with either TGF-β or IL6 reprogrammed normal fibroblasts to an activated state, which resembled that of patient-derived CAFs [35]. Notably, this transition was accompanied by the modulation of selected sets of miRNAs, including up-regulation of miR-210 and miR-133b (Fig. 1B) [35]. Both miRNAs were shown to be able *per se* to promote fibroblast activation when ectopically over-expressed in normal fibroblasts [35,36], thus suggesting their pivotal role in the establishment of a CAF-phenotype, and consequently, of the ability to fuel cancer cell aggressiveness.

MiRNA changes occurring in non-tumor cells may dramatically influence metastasis even systemically. For instance, it has been shown that miR-155 deficiency in hematopoietic cells promoted macrophage infiltration in lungs and enhanced lung metastasis in tumor-bearing mice, although without obvious influence on primary tumor progression (Fig. 1B) [37]. Specifically, the authors performed bone marrow transplantation using miR-155–deficient mice as donors and wild-type mice as recipients, and inoculated chimeric mice with tumor cells. Deletion of miR-155 in the bone marrow resulted in increased recruitment and polarization of macrophages in the lungs, thus promoting seeding, extravasation, and persistent growth of tumor cells at metastatic sites [37].

### 3.2. Extracellular miRNAs as novel signaling molecules

We have previously summarized how miRNA expression and function may be influenced in either tumor or microenvironmental cells by reciprocal stimulation, usually through secretion of cytokines. In the last years, it has become evident that miRNAs themselves may act as transferrable messages, thus playing roles as autocrine, paracrine, and endocrine signaling molecules, even at long distance [38]. Extracellular miRNAs were shown to be incredibly stable in body fluids (and cell culture media), suggesting that they are somehow protected from RNAase action through binding to proteins or encapsulation within vesicles [39]. Extracellular vesicles (EVs) are defined as spherical bilayered membrane vesicles released by multiple cell types, which contain specific repertoires of lipids, proteins, and non coding RNAs, including miRNAs [40]. Their cargo is transferable from donor to recipient cells, where it is functionally bioactive. For example, EV-transferred miRNAs were shown to efficiently down-regulate their target mRNAs in recipient cells. Current criteria to distinguish between diverse EV populations are based on size, density, subcellular origin, function and molecular cargo. Basically, EVs may be classified into exosomes (30–100 nm diameter), microvesicles (100–1000 nm diameter) and large oncosomes (1–10 mm diameter) [40]. Exosomes are formed from inward budding of the endosomal membrane, whereas microvesicles originate directly from the plasma membrane, and are often classified as ectosomes. Large oncosomes originate from the shedding of membrane blebs and are usually released by tumor cells that acquired amoeboid phenotype, which consists of an alternative mechanism of tumor cell dissemination as compared to the mesenchymal one [41,42]. Also apoptotic bodies, 1 μm–5 μm-sized vesicles released by apoptotic cells, are categorized as EVs by some authors [40].

Among EVs, exosomes are certainly the most studied, though it must be noted that current methodology does not allow proper separation or discrimination of the different EV types of similar sizes, nor the isolation of pure fractions of them (for this reason, we will use the generic term EV in subsequent paragraphs). It has been shown that, in general, miRNAs are enriched in cancer exosomes when compared with normosomes (*i.e.* exosomes from normal cells), and in exosomes derived from metastatic cancer cells when compared to those from non-metastatic cells [43]. Enrichment of miRNAs in exosomes from a given cell type/state is not a mere reflection of the donor cell composition but rather the result of a regulated, but still largely unresolved, sorting mechanism. In fact, though several studies have found that the bulk of miRNAs released in exosomes reflects the cellular miRNA expression profile and the majority of miRNAs (about two-thirds) are released from cells passively by mass action, a subset of miRNAs is overrepresented in exosomes, meaning that they are enriched in comparison to levels observed in the cell, and others are underrepresented, indicating a selective release mechanism [44]. In a global profiling study, it has been shown for example that Ago2 may play a role in such selective pathway targeting miRNAs to EVs [45]. Another intriguing aspect is that cancer exosomes are not just boxes where mature miRNAs may be loaded but are sites of cell-independent miRNA biogenesis. It has been indeed reported that breast cancer associated exosomes contain miRNAs associated with Ago2, Dicer and TRBP and display intrinsic capacity to process pre-miRNAs into mature molecules [43].

Though several reports showed that EVs may be the carriers of extracellular miRNAs, it is not yet clear which could be the prevalent form. For example, some authors showed that the concentration of miRNAs from serum or saliva was consistently higher in the exosome pellet compared to the exosome-depleted supernatant [46]. In another study, it has been instead reported that extracellular miRNAs are predominantly EV-free and are associated with Ago proteins [39]. The authors hypothesized that extracellular miRNAs are in the most part by-products of dead cells that remain in extracellular space due to the high stability of the Ago2-miRNA complex, and that only a small proportion of circulating miRNAs are enclosed in EVs [39].

Whether they are EV–associated or bound to proteins, extracellular miRNAs have been undeniably shown to reprogram the transcriptome of target cells. For example, metastasized tumor cells may phenocopy their metastatic behavior to less malignant cells at the primary tumor site through the long-range transfer of miR-200-enriched EVs (Fig. 1B) [47]. By delivering extracellular miRNAs, tumor cells may also influence the activity of non-tumor cells, such as macrophages or endothelial cell [48,49]. In this regard, miR-21 and miR-29a, enriched in exosomes of lung cancer cells, were shown to function as paracrine agonists of Toll-like receptors in tumor-adjacent macrophages, thus inducing a pro-inflammatory cascade that, through the release of TNF-α and IL6 by immune cells, ultimately promoted cancer growth and dissemination (Fig. 1B) [48]. It has been also reported that cancer-secreted exosome-associated miR-105 is efficiently taken up by endothelial cells as a mature miRNA (in fact no changes in miR-105 endogenous transcription were observed in recipient cells), where it destroys tight junctions by targeting ZO1 (Fig. 1B) [49]. By altering the integrity of these natural barriers against metastasis and enhancing vascular permeability, EV-derived miR-105 was shown to exert a dual metastasis-promoting effect by i) favoring the intravasation of metastatic cells at the primary site and ii) the formation of a pre-metastatic niche at secondary sites, thus facilitating extravasation of disseminated cancer cells [49]. Non-tumor cells in the premetastatic niche have been also shown to be reprogrammed in their metabolism by miR-122-enriched breast cancer EVs (Fig. 1B) [50]. By down-regulating the

glycolytic enzyme pyruvate kinase in recipient cells, miR-122 is in fact able to systemically suppress glucose utilization by non-tumor cells to increase nutrient availability for cancer cells in target organs, such as brain and lungs [50], thus ultimately favoring metastasis.

The examples provided so far showed how tumor cells may condition their microenvironment through the release of extracellular miRNAs. However, it must be noted that even stromal cells may use miRNAs as signaling molecules to influence tumor behavior. In this regard, it was shown, for instance, that prostate CAFs can release miR-133b in the extracellular space, where it seems to be predominantly not associated to EVs [35]. Soluble miR-133b was shown to be efficiently taken up by prostate cancer cells, where it may contribute to establish a mesenchymal phenotype (Fig. 1B) [35]. This represents a potential additional mechanism by which CAFs can induce EMT in tumor cells, through the direct transfer of miRNAs typically expressed in cells of the mesenchymal lineage. Moreover, it is likely that released miR-133b may act as a paracrine stimulus able to expand the reactive phenotype to adjacent fibroblasts, thus contributing to extend the stromal niche able to support cancer progression [35]. Another example of stromal-derived extracellular miRNA is represented by astrocyte-released exosome-associated miR-19 (Fig. 1B) [51]. By transferring miR-19, astrocytes induce PTEN suppression in tumor cells that have metastasized to the brain, leading to an increased secretion of the chemokine CCL2, which recruits IBA1-expressing myeloid cells that reciprocally enhance the outgrowth of brain metastatic cancer cells via enhanced proliferation and reduced apoptosis [51].

An additional possible intriguing function of EV-associated miRNAs could be the chance for tumor cells to discard tumor suppressive miRNAs by sequestering them into vesicles. In this context, it has been shown that EVs obtained from the serum of patients with thyroid cancer were significantly enriched with miR-145, a miRNA able to inhibit cancer cell proliferation, migration and invasion, and induce apoptosis through the repression of PI3K/AKT pathway, which is usually down-regulated in thyroid cancer compared to normal cells [52].

The emerging scenario is hence that extracellular miRNAs do play a crucial role in the complex reciprocal communication between tumor cells and their microenvironment, either at the primary or at the metastatic site. The peculiar stability of extracellular miRNAs in body fluids allows a fairly easy interception of the ‘message in transit’, thus stimulating the interest towards these molecules also as novel diagnostic, prognostic, and predictive biomarkers.

### 3.3. miRNAs regulating metastatic tropism: the bone disease

A salient feature of metastasis is the diversity of signaling mediators that enable tumor cells of different origin to metastasize to a specific organ. The skeleton represents a common site of metastases for osteotropic tumors, such as breast [53] and prostate [54] cancers. Metastatic bone disease arises as a result of a complex process characterized by a dynamic crosstalk between the primary cancer and the bone. In fact, through the release of soluble factors and EVs the primary lesion contributes to prepare a fertile soil, thus establishing a pre-metastatic niche before colonization by cancer cells [55].

Bone is a complex tissue composed of multiple cell types including osteoblasts and osteoclasts, which operate in the finely tuned processes of bone formation and resorption, respectively [56]. Breast cancer bone metastases are mostly osteolytic, characterized by bone degradation as a result of an increased osteoclast activity, which is primarily regulated through the receptor activator of nuclear factor kB (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) axis [57]. Osteolysis is based on a self-perpetuating signaling system (the “vicious cycle”) that involves mitogenic factors for tumor cells, including TGF-β, insulin like growth factor-1 (IGF-1), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), and calcium released from the bone matrix as well as tumor-derived parathyroid hormone-related peptide (PTHrP) [58]. Conversely, prostate cancer bone metastases are predominantly osteoblastic, characterized by excessive bone formation resulting from augmented osteoblast activity [54]. Once cancer cells reach the bone, the type of metastatic lesion that will develop mainly relies on the cancer cell phenotype and the specific interaction with various cellular and molecular components of bone microenvironment. In this context, the interaction between the chemokine (C-X-C motif) receptor (CXCR) 4 (CXCR4), expressed on cancer cells, and its ligand stromal derived factor 1 (SFD1), appears critically important in the formation of both prostate and breast cancer bone metastasis [59]. It has been suggested that tumor cells must adapt to the bone environment for the development of overt metastasis. This adaptation requires that tumor cells express genes that are normally expressed by bone cells in a process called osteomimicry [60].

The variety of molecular factors thought to be involved in the specific bone tropism of cancer cells includes miRNAs. Indeed, a number of miRNAs known to be aberrantly expressed in breast and/or prostate cancer was shown to possibly contribute to metastatic bone disease, although their precise role in organ-specific metastasis is far from being understood. As far as breast cancer is concerned, functional studies demonstrated that overexpression of miR-126, a miRNA down-regulated in the disease, strongly suppressed the metastatic colonization of breast cancer cells by inducing a cell-extrinsic modulation of the microenvironment (Fig. 2). Specifically, the miRNA impaired the ability of breast cancer cells to recruit endothelial cells to incipient metastasis *in vivo* through direct targeting of pro-angiogenic and metastatic genes such as insulin-like growth factor binding protein 2 (IGFBP2), phosphatidylinositol transfer protein, cytoplasmic 1 (PITPNC1) and c-mer proto-oncogene tyrosine kinase (MERTK) [61]. In addition, miR-126 was found to decrease the formation of breast cancer bone metastasis by targeting the matrix metallopeptidase 13 (MM13) [62] and the vascular endothelial molecule-1 (VCAM-1) [63].

miR-218 was identified as a pro-metastatic miRNA as it favors the up-regulation of bone sialoproteine (BSP), osteopontin (OPN) and CXCR4 in breast cancer cells (Fig. 2) [64]. In addition, it was found to promote osteomimicry through WNT pathway activation in bone-homing metastatic breast cancer cells [65]. Through the analysis of miRNAs differentially expressed between parental MDA-MB-231 breast cancer cells and a highly bone metastatic MDA-MB-231 variant, miR-204, miR-211 and miR-379 –the expression of which was decreased in the metastatic cell line– were identified as negative regulators of the TGF-β-induced expression of IL11, a key pathogenetic process in breast cancer metastasis [66]. Reconstitution of miR-141 and miR-219, two miRNAs directly associated with cancer-induced osteolytic bone disease and significantly down-regulated in osteoclasts during cancer-stimulated osteoclast differentiation, were found to decrease osteolytic breast cancer bone metastases likely through the impairment of osteoclast activity and the prevention of bone metastatic resorption (Fig. 2) [67]. Two additional miRNAs, miR-203 and miR-135, were found to reduce breast cancer bone metastases via targeting the bone-specific effector Runx2 [68].

As regards prostate cancer, analysis of miRNA expression profiles in clinical samples of primary and bone metastasis identified miR-143 and miR-145 as significantly down-regulated in bone lesions (Fig. 2). Consistently, ectopic expression of the miRNAs in prostate cancer cells reduced tumor progression and bone invasion *in vivo*, thus suggesting miR-143 and miR-145 as suppressors of bone metastasis in the disease [69]. miR-203 was also proposed as a suppressor of prostate cancer bone metastasis. Specifically, it was found that low miR-203 expression levels correlated with prostate cancer progression, and that miRNA reconstitution in prostate cancer cells decreased their ability to metastasize in a mouse model of bone metastasis [70]. Similarly, overexpression of miR-25, a miRNA strongly decreased in highly osteotropic cancer stem/progenitor subpopulation of human prostate cancer cells and directly regulating integrin-αv expression, was found to reduce the metastatic dissemination to the bone, possibly via attenuation of extravasation *in vivo* [71]. Again, ectopic expression of

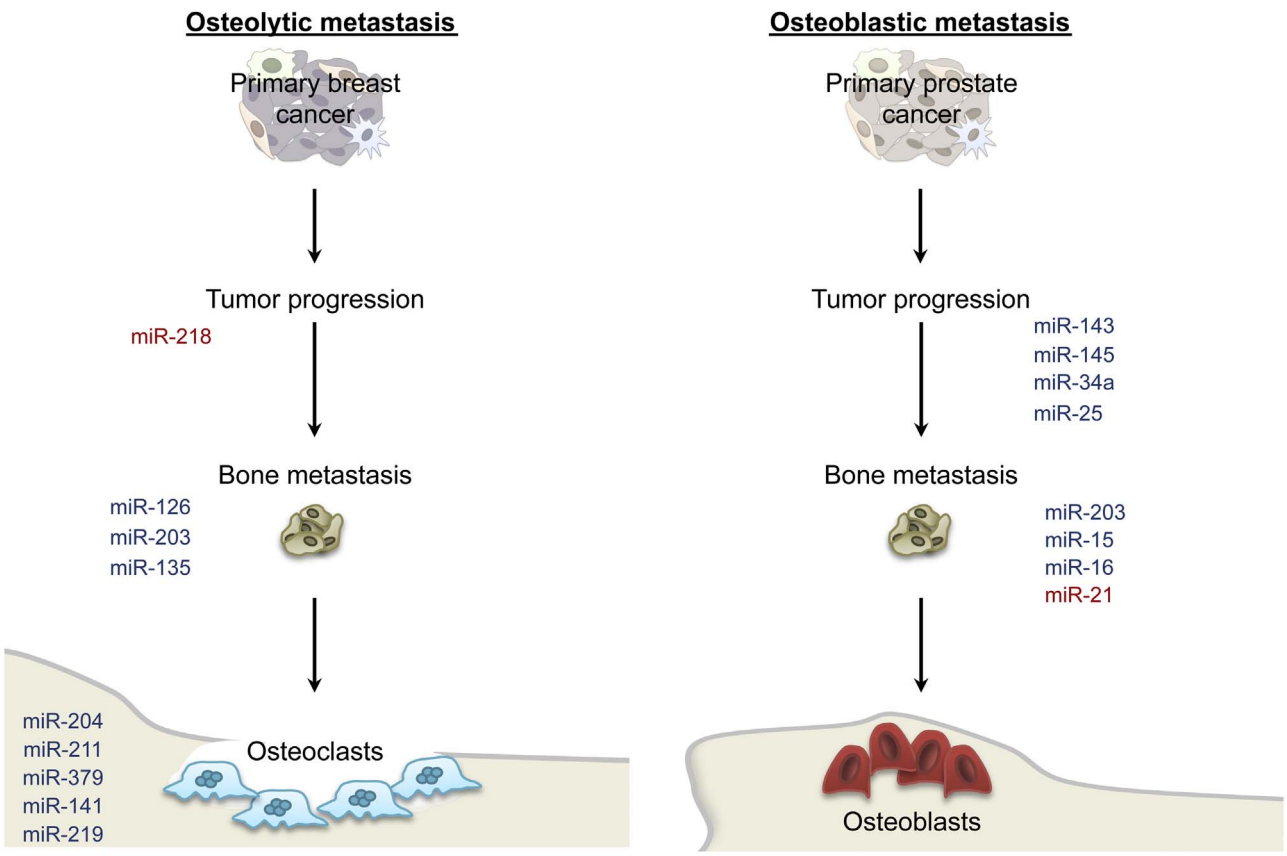


**Fig. 2.** miRNAs involved in the control of metastatic bone disease in breast and prostate cancer. Illustrated are osteolytic and osteoblastic lesions, frequently observed in breast and prostate cancer, respectively. Specific miRNAs are depicted in the figure based on their documented role in different stages of the bone metastasis process: pro-metastatic and anti-metastatic miRNAs are highlighted in red and blue, respectively. We refer the reader to the main text ("miRNAs regulating metastatic tropism: the bone disease") for more detailed information on the individual miRNAs.

miR-34a inhibited bone metastasis in a RAS-dependent prostate cancer xenograft model through direct targeting of the WNT signaling-related gene TCF7 [72]. Recently, it has been shown that loss of miR-15 and miR-16 in cooperation with increased miR-21 expression endows bone-metastatic potential to prostate cancers (Fig. 2). Indeed, this combination of miRNAs was able to promote distant bone marrow colonization and osteolysis in prostate cancer models possibly through aberrant activation of TGF-β signaling [73]. These findings are, at least in part, consistent with previous evidence indicating that systemic delivery of miR-16 with atelocollagen in mice significantly inhibited prostate cancer cell growth in bone [74].

## 4. Metastamirs: new targets for treatment of cancer metastasis?

Experimental evidence of a direct involvement of specific miRNAs in the dissemination process has aroused interest in the development of miRNA-based strategies for the prevention and treatment of metastasis [75]. In this context, miRNAs exerting either pro-metastatic (miR-21) or anti-metastatic functions (miR-15/16, miR-34a, miR-132, miR-203, miR-205) by interfering with two or more steps of the metastatic cascade, appear particularly appealing for the development of novel therapeutic approaches, although the precise identification of relevant miRNA targets in specific tumor types still needs to be accurately defined in translatable preclinical models of spontaneous metastasis.

The expression of miRNAs that are down-regulated/absent in tumor cells can be restored through an approach −referred to as "miRNA replacement therapy"– based on the use of synthetic double-stranded RNA oligonucleotides able to mimic native miRNAs [76]. Such molecules, which contain chemical modifications to guarantee improved stability and cellular uptake, are designed to allow the exclusive production of the mature miRNA and to preserve the interaction with the natural targets. On the other hand, miRNAs that are over-expressed in tumor cells can be inhibited using single-stranded, chemically modified antisense oligonucleotides (mainly locked nucleic acids, LNAs), that work as selective miRNA inhibitors by annealing to the mature miRNA and inhibiting the interaction with its targets [76]. Another inhibitory strategy relies on the use of "miRNA sponges", RNA molecules containing multiple tandem binding sites for the miRNA of interest, which are able to block miRNA function through competition with endogenous target transcripts [76]. Finally, "miR-masks" are single stranded, chemically modified oligonucleotides that act as gene-specific miRNA inhibitors by preventing the interaction of the miRNA with a specific target mRNA [76].

Preclinical research carried out in mice is encouraging towards translating miRNA-based strategies into human cancer therapy based on the observed therapeutic activity in the absence of major adverse events. However, further improvements in terms of efficacy and targeted delivery to the tumor are still necessary. In this context, approaches to promote specific homing of the miRNA modulator to tumor cells have been developed based on the conjugation of the therapeutic oligonucleotides with targeting molecules, such as peptides, antibodies or other active molecules [77]. In addition, novel nanotechnology-assisted delivery systems that hold potential for next-generation miRNA-based therapeutics are currently being investigated [78].

Interestingly, the applicability of strategies aimed at modulating specific miRNA expression in the clinical setting is currently under investigation. Specifically, in 2013, an miR-34 mimic encapsulated in lipid nanoparticles (MRX34) entered a multicentre Phase I clinical trial in patients with various solid tumors, showing evidence of antitumor activity in a subset of patients with refractory tumors [79]. However,

owing to immune-related severe adverse events, the trial was terminated. More recently, an miR-16 mimic delivered in nanocells with EGFR antibody surface conjugation (MesomiR-1) entered a Phase I clinical trial in patients with malignant pleural mesothelioma or non-small cell lung carcinoma [80]. In addition, a phase I trial with a LNA-based antimiR-155 (MRG-106) has recently initiated in patients with cutaneous lymphoma and mycosis fungoides.

As far as non-oncologic diseases are concerned, in a Phase II clinical trial, the chronic administration of an LNA-modified anti-miRNA against miR-122 (Miravirsen), developed for the treatment of hepatitis C (HCV), has demonstrated to be an effective and safe treatment strategy for patients with chronic HCV genotype 1 infection [81]. However, it has been found recently that long term *in vitro* treatment with Miravirsen induced resistance due to mutations in the viral genome [82]. The emergence of resistance could also represent a problem for miRNA-based antitumor approaches. In this context, we recently demonstrated that a persistent activation of ERK1/2 and AKT in miR-34a-reconstituted human peritoneal mesothelioma cell lines was able to counteract the anti-proliferative and pro-apoptotic effects of the miRNA, possibly representing a resistance mechanism [83].

An additional problem that can be envisaged for the translation of miRNA-based therapeutics into the clinic is related to miRNA redundancy (*i.e.*, the regulation of a single target mRNA by several miRNAs) and the consequent possibility that targeting an individual miRNA may not be sufficient to get a therapeutic effect. Recent results obtained in experimental models indicate that co-treatment of tumor cells with selective inhibitors of different miRNAs, such as for example miR-21 and miR-10b [84], or concomitant replacement of different miRNAs, such as miR-137 and miR-197 [85], produced significant antitumor effects. In addition, experimental evidence has been provided that targeting a pro-metastatic miRNA, such as miR-21, can also enhance the tumor response to anticancer drugs [86].

Overall, miRNA-based therapeutics hold great promise as highly specific, targeted therapies for cancer treatment. However, to accelerate their adoption in the clinic, there still exists the need to improve their chemical designs and to develop more specific and efficient delivery strategies in order to guarantee prolonged therapeutic efficacy and long-term safety *in vivo*.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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