microRNA: New Players in Metastatic Process

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1. Introduction

In the last years new players have been revealed in cancer biology: microRNA (miRNAs or miRs) a class of small non coding RNAs (19-22 nts) able to regulate gene expression at post-transcriptional level, binding through partial sequence homology mainly the 3′ UTR of target mRNAs, and causing block of translation and/or mRNA degradation.

miRNAs are generated by an endogenous transcript, they represent approximately 1% of the genome of different species, and each of them has hundreds of different conserved or non conserved targets: it has been estimated that about 30% of the genes are regulated by at least one miRNA. miRNA genes, expressed in several organisms, including *Homo Sapiens*, are highly conserved across different species [1].

This discovery resulted in a pattern shift in our understanding of gene regulation because miRNAs are now known to repress thousands of target genes and coordinate normal processes, including cellular proliferation, differentiation and apoptosis. They are highly specific for tissue and developmental stage, and play crucial functions in the regulation of important processes, such as development, and stress response. In the last few years, miRNAs have indeed taken their place in the complex circuitry of cell biology, revealing a key role as regulators of gene expression.

In 2002, Croce and colleagues first demonstrated that a miRNA cluster was frequently deleted or downregulated in chronic lymphocytic leukemia. This discovery suggested that non-coding genes were contributing to the development of cancer, and paved the way for a closer investigation of miRNA loss or amplification in tumors.

miRNAs expression profiling has indeed provided evidence of the association of these tiny molecules with tumor development, progression and response to therapy, suggesting their possible use as diagnostic, prognostic and predictive biomarkers. It has been demonstrated that miRNAs can act either as oncogenes or tumor suppressors, and more recently it has

been demonstrated that a miRNAs can exploit both functions according to the cellular context of their target genes. Another important issue concerns the role of miRNAs in regulating the interaction between cancer cells and the microenvironment with respect to neo-angiogenesis or tissue invasion and metastasis.

Outgrowths of disseminated metastases remain the primary cause of mortality in cancer patients, but the molecular and cellular mechanisms regulating metastatic spread remain largely unknown. Metastatic processes involve multiple steps, including detachment from primary tumors, crossing the basement membrane barriers and extracellular matrix, intravasation into the circulation, survival within the vasculature, extravasation into distant tissues, and finally, establishment of secondary tumors [2] . These processes rely on coordinated spatio-temporal expression of various genes and finely regulated protein products, which govern the ability of tumor cells to successfully complete the intricate task, and the pivotal role of miRNAs in metastasis has emerged only recently.

2. miRNAs biogenesis and mechanism of action

miRNAs are generated by endonucleolytic cleavage of hairpin precursor transcripts by Dicer ribonuclease (RNase) III–like proteins and can direct the cleavage of target transcripts by Argonaute RNAse H–like proteins in a sequence-specific manner. miRNAs can also inhibit translation of target mRNAs.

miRNAs are transcribed for the most part by RNA Polimerase II as long primary transcripts characterized by hairpin structures (pri-microRNAs), and part of them are transcribed as distinct transcriptional units. 50% of known miRNA genes are located nearby other microRNAs, supporting the hypothesis that clustered miRNAs, representing miRNA families which are commonly related in sequence and function, can be transcribed from their own promoters as polycistronic pri-microRNAs.

According to their genomic localization, microRNAs can be classified in:

a) exonic microRNAs located in non coding transcripts, b) intronic microRNAs located in non coding transcripts and microRNA located in protein-coding trancripts, c) mixed miRNA genes that can be assigned to one of the above groups depending on the given splicing pattern. Exonic microRNAs are transcribed within the pri-miR (up to 1 kb long) containing both the 5'-cap and the 3'-poly-A tail. The miRNAs localized within introns of protein-encoding or -non-encoding genes have been denominated "miRtrons". miRtrons are regulatory RNAs transcribed within the mRNA of the host gene generating a hairpin structure, recognized and cleaved by the spliceosome machinery without Drosha-mediated cleavage.

The initial step in pri-miRNA processing (Figure 1) is performed in the nucleus by the enzymatic activity of an RNAse III-type protein called Drosha. Drosha is a highly conserved 160 kDa protein containing two RNAse III domains and one double-strand RNA-binding domain. Drosha forms a huge complex, 500 kDa in *D. melanogaster* and 650 kDA in *H. sapiens*, known as Microprocessor complex, which generates a ~70-nucleotides precursor

miRNA (pre-miRNA) and contains the co-factor Di George syndrome critical region 8 (DGCR8), also known as Pasha in *D. melanogaster* and *C. elegans.*
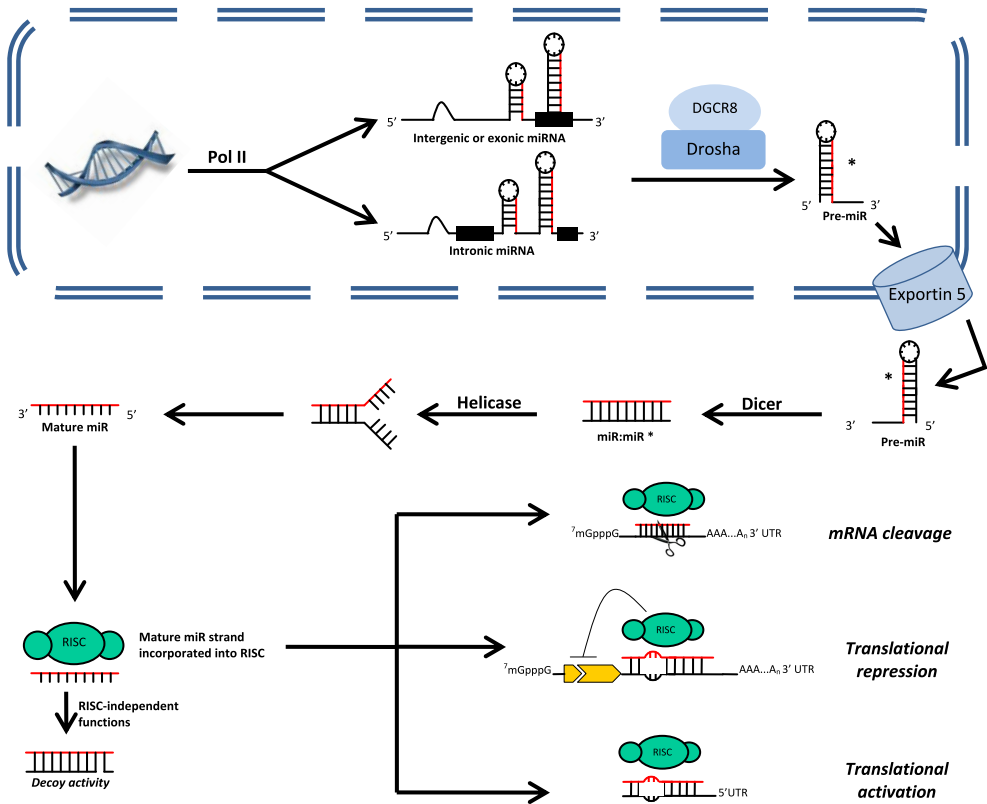

Figure 1. An overview of microRNAs biogenesis and mechanism of action.

The originated precursor molecules are then actively exported by a Ran-GTP and Exportin 5-mediated mechanism to the cytoplasm, where the second step of pre-miRNA processing (dicing) is mediated by the RNAse III Dicer (~200 kDa), which acts in complex with the transactivating response RNA-binding protein (TRBP), or PACT (also known as PRKRA), and Argonaute (AGO1-4), generating a dsRNA of approximately 22 nucleotides, named miR:miR*. This dsRNA includes the mature miRNA guide, and the complementary passenger strand, the miRNA* (star miRNA) (many publications refer to the two strand pair as miR-3p/miR-5p, referring to the direction of the functional miRNA). Whereas one of the two strands is selected as guide strand according to thermodynamic properties, the complementary one is usually subjected to degradation. The so called miRNA* was initially thought to be the strand subjected to degradation, instead more recent evidence suggests that it does not simply represent a non-functional bioproduct of miRNA biogenesis, but it can be selected as a functional strand and play significant biological roles [3] .

More in details, guided by the sequence complementarity between the small RNA and the target mRNA, miRNA-RISC-mediated gene inhibition is commonly divided into three processes: (i) site-specific cleavage, (ii) enhanced mRNA degradation and (iii) translational inhibition. The first process, commonly defined as RNA interference (RNAi) and restricted to miRNAs with a perfect or near-perfect match to the target RNA, is a very rare event in mammals, where it is carried out exclusively by Ago2. By contrast, the other two processes are more commonly associated with mismatched miRNA/target sequences, which is the most likely scenario in mammals. The combination of these two processes is commonly defined as a non-cleavage repression, and can be carried out by any of the four mammalian Ago proteins [4] . However, the exact mechanism through which miRNAs can impair translation is still debated.

Moreover, even though it is known that microRNAs mainly recognize complementary sequences in the 3' untraslated regions (UTRs) of their target mRNAs, more recent studies have reported that they can also bind to the 5'UTR or the ORF [5-8] and, even more surprisingly, they can upregulate translation upon growth arrest conditions [9] .

Finally, whereas the 5' end of the microRNA (the so called "seed site") has always been considered the most important for the binding to the mRNA, recently the target sites have been further divided into three main classes, according to grade and localization of the complementarity [10] : the dominant seed site targets (5' seed-only), the 5' dominant canonical seed site targets (5' dominant) and the 3' complementary seed site targets (3' canonical).

Considering the different rules regulating the interaction between a microRNA and its target mRNA, it is not surprising that each miRNA has the potential to target a large number of genes [11-14]. Conversely, an estimated 60% of the mRNAs have one or more evolutionarily conserved sequences that are predicted to interact with miRNAs. Bioinformatical analysis predicts that the 3' UTR of a single gene is frequently targeted by several different miRNAs [11] . Many of these predictions have been validated experimentally, suggesting that miRNAs might cooperate to regulate gene expression (a list of computational tools for miRNA target prediction is reported in Table 1).

name	website
miRNA map	http://mirnamap.mbc.nctu.edu.tw/
miRBASE	http://mirbase.org/
microRNA	http://www.microrna.org/microrna/home.do
coGemiR	http://www.cogemir.tigem.it/
miRGEN	http://www.diana.pcbi.upenn.edu/miRGen.html
deepBase	http://www.deepbase.sysu.edu.cn

Table 1. miRNA databases.

To complicate the already intricate scenario, it has been recently reported that miRNAs can bind to ribonucleoproteins in a seed sequence and a RISC-independent manner and then interfere with their RNA binding functions (decoy activity) [15] . Three studies have reported that miRNAs can also regulate gene expression at the transcriptional level by direct binding to the DNA [16-18].

Overall, these data show the complexity and widespread regulation of gene expression by miRNAs that should be taken into consideration when developing miRNA-based therapies.

3. Metastasis

The most deleterious effect of cancer is metastases development, indeed tumor metastasis is the primary cause of death in cancer patients. The ability to metastasize is a hallmark of malignant tumors [19] . Metastases represent the end point of a multi-step process that consists of local invasion through surrounding extracellular matrix and stromal cells, intravasation into the blood vessels, survival in the circulation, extravasation, and colonization of distant tissues [20]. Each step in this process represents a physiological barrier that must be overcome by the tumor cell to successfully metastasize. Malignant cells overcome these barriers through the accumulation of genetic and epigenetic changes, including modifications in microRNA expression profiles. Despite great improvement in the knowledge of metastasis biology, the molecular mechanisms which underlie this intricate process are still not completely understood.

Tumor cells can invade surrounding tissues as cohesive multicellular units or as individual cells, and individual cells can invade through the 'amoeboid invasion' or the 'mesenchymal invasion' programs [21] . Amoeboid movement depends mainly from Rho/ROCK expression, and is independent from adhesion and proteolytic degradation of ECM [22,23]. On the contrary, mesenchymal motility depends upon interaction of carcinoma cells with the extracellular matrix through integrin recruitment and upon pericellular ECM proteolysis of the moving cells. Cells that use this program to invade are characterized by an elongated and polarized morphology, achievable with an epithelial to mesenchymal transition (EMT). EMT, first described as typical of embryonic development, generates cells with mesenchymal features phenotypically similar to invading cells. The EMT transcriptional programme has been associated with activation of several key transcriptions factors, including Snail1 and Snail2 (Slug), Twist, ZEB-1-2, etc, which lead to the regulation of a series of proteins causing decrease of E-cadherin for disruption of adherent junctions, increase in N-cadherin and Met proto-oncogene to drive motility, as well as increase in MMPs and urokinase–type plasminogen activator/urokinase–type plasminogen receptor (uPA/uPAR) proteolytic systems to degrade 3D barriers [24,25].

The overexpression of many of these EMT regulators have been shown to correlate with disease relapse and decreased survival in patients with breast, colorectal, and ovarian carcinomas, suggesting that the induction of EMT leads to more aggressive tumors and poorer clinical outcomes.

Once tumor cells have invaded local microenvironment, they should intravasate, survive in the circulation and extravasate at distant sites. To successfully perform these steps, they have to cross the pericyte and endothelial cell barriers that form the walls of microvessels. In order to overcome physical barriers which represent an obstacle to extravasation in tissues with low intrinsic microvessel permeability, primary tumors are capable of secreting factors that perturb these distant microenvironments and induce vascular hyperpermeability. The ability of the cancer cell to develop into a metastatic lesion at distant sites is the most limiting step in cancer metastasis formation. Indeed, the disseminated tumor cells may stay in a quiescent state for long time, probably due to incompatibilities with the foreign microenvironments that surround them [2]. Some have proposed that carcinoma cells can address the problem of an incompatible microenvironment at the metastatic site via the establishment of a "premetastatic niche" [26].

4. miRNAs and metastasis

Remarkably, a regulatory role for miRNAs in metastasis has been recognized, and the term "metastamir" has been coined by Welch and colleagues to refer to those regulatory miRNAs not just involved in tumorigenesis, but specific in the promotion or suppression of various steps of metastasis [27]. To date, microRNAs have mostly been found to influence the initial stages of metastasis, affecting cell migration and invasion (Figure 2). Although a particular miRNA that specifically regulates cancer cell intravasation and extravasation has not yet been identified, it is still believed that these steps may also be regulated by miRNAs.
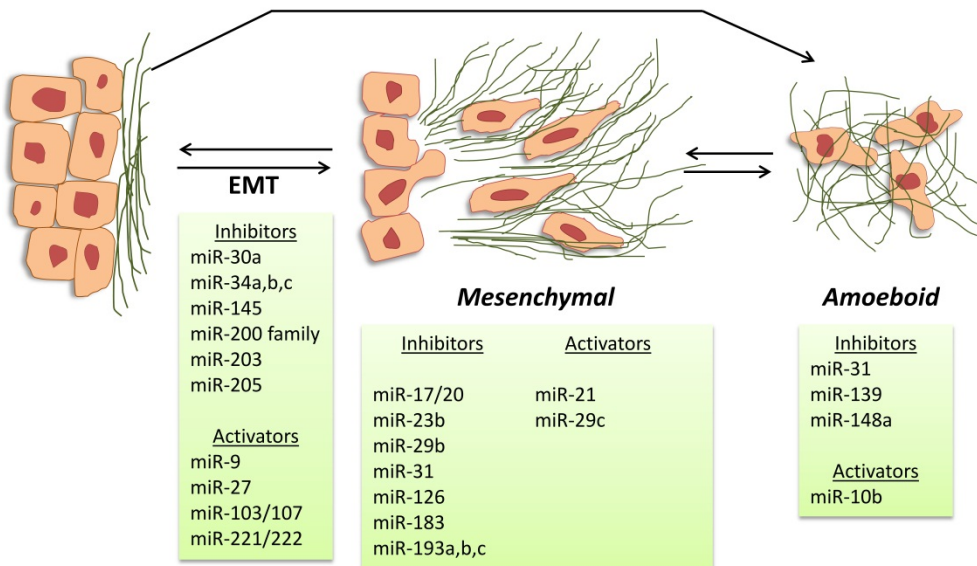

Figure 2. microRNAs implicated in the regulation of EMT and cell migration.

Several miRNAs have been found to regulate the EMT process, and the most well-known among them is the miR-200 family, which includes miR-200a, miR-200b, miR-200c, miR-141 and miR-429.

miR-200 family is recognized as a master regulator of the epithelial phenotype by post transcriptionally suppression of the expression of the ZEB1 and ZEB2 EMT-inducing transcription factors in breast [28] and gastric cancer [29]. Acting in the opposite direction, ZEB1 and ZEB2, which promote not only tumor cell dissemination, but also the tumor-initiating capacity, has been shown to transcriptionally repress miR-200 family members, thereby establishing a double negative feedback loop that causes the reinforcement of cells in either the mesenchymal or epithelial state [25]. This miR family, as others able to control epithelial–mesenchymal plasticity, is likely to also affect events at metastatic sites. Recently, the putative DNA methylation-associated inactivation of various miR-200 members has been described in cancer. miR-200 epigenetic silencing resulted to be not a static and fixed process, instead there can be a shift to hypermethylated or unmethylated 5'-CpG island status corresponding to the EMT and mesenchymal-epithelial-transition (MET) phenotypes, respectively. In fact, careful laser microdissection in human colon revealed that in normal colon mucosa crypts (epithelia) and stroma (mesenchyma) 5'-CpG island status are unmethylated and methylated at these loci, respectively, and that the colorectal tumors undergo selective miR-200 hypermethylation of their epithelial component. These findings indicate that the epigenetic silencing plasticity of the miR-200 family contributes to the evolving and adapting phenotypes of human tumors [30].

Unexpectedly, it was reported that overexpression of miR-200 enhances macroscopic metastases in mouse breast cancer models. These findings were surprising but provide yet another example of the opposing activities of some miRNAs [31]. miR-200 levels are indirectly downregulated by miR-103/107 that target Dicer, a key component of the miRNA processing machinery. Accordingly, miR-103/107 are associated with metastasis and poor outcome in human breast cancer [32].

The transcription factor ZEB1 can also repress the expression of stemness-inhibiting miR-203 [33]. Recently, miR-203 was reported as a metastasis suppressor miRNA, targeting Slug [34] and Snail1 [35] and is often silenced in different malignancies including hepatocellular carcinoma, prostate cancer, oral cancer, breast cancer and hematopoietic malignancy. Snail1 and Slug play a key role during the early step of EMT, activating expression of ZEB factors in a context-dependent manner. Functionally, ectopic expression of miR-203 in BT549 and MDA-MB-231 breast cancer cell lines caused cell cycle arrest and apoptosis and inhibited cell invasion and migration *in vitro*. Thus the miR-203 and miR-200 feedback loops control cell plasticity in epithelial homeostasis. Snail1 is also regulated by miR-30a in non-small cell lung cancer (NSCLC), where it is dowmodulated [36].

Opposite to miR-200 family, miR-221/222 family promotes a poorly differentiated mesenchymal-like phenotype in breast cancer, and is highly expressed in triple negative breast cancers that basally expressed EMT markers. Increasing miR-221 or miR-222 can affect various characteristics associated with EMT, including increased invasive capacity,

[37,38], and anoikis resistance [39]. Forced expression of miR-221/222 in luminal breast cancer cells causes a decrease in E-cadherin and an increase in the mesenchymal marker vimentin [40]. Luminal cells expressing miR-221/222 gained a more mesenchymal morphology and had increased migratory and invasive capacity [41]. Furthermore, miR-221 and miR-222 can regulate angiogenesis, repressing the proliferative and angiogenic properties of c-Kit in endothelial cells [42]. In addition other miRNAs can manage EMT, such as the ZEB1- and ZEB2-suppressing miR-205 [28], which has also been shown to exert an oncosuppressive activity in breast cancer [43,44] prostate cancer [45] and melanoma [46]; and miR-27, which promotes EMT in gastric cancer cell directly targeting APC gene and activating the Wnt pathway [47].

Recently, the inhibition of EMT by p53 has been described as a new mode of tumor suppression which presumably prevents metastasis. p53 activation down-regulates Snail via induction of the miR-34a/b/c genes, which directly target Snail transcription factor. Ectopic miR-34a expression caused down-regulation of Slug and ZEB1, as well as the stemness factors BMI1, CD44, CD133, OLFM4 and c-MYC, thus provoking MET. Conversely, the transcription factors Snail and ZEB1 repress miR-34a and miR-34b/c expression [48]. Recently it has been described that miR-34 suppress also c-MET in hepatocellular carcinoma [49] and in osteosarcoma cells [50].

EMT is characterized by cadherin switching (from E-cadherin to N-cadherin), that correlates with a profound change in cell phenotype and behavior. miR-9, identified as a new "metastomiR" and activated by MYC and MYCN, directly targets CDH1, the E-cadherin-encoding messenger RNA, leading to increased cell motility and invasiveness, activation of β-catenin signaling and upregulation of VEGF. Moreover, overexpression of miR-9 in non-metastatic breast tumor cells enables these cells to form pulmonary micrometastases in mice, and in colorectal cancer cells it promotes cell motility [51]. Conversely, inhibition of miR-9 in highly malignant cells impairs metastasis formation [52].

N-cadherin (CDH2) was proved to be a direct target of miR-145 by Gao P and coworkers [53]. miR-145, suppressing N-cadherin protein translation and indirectly downregulating also its downstream effector matrix metallopeptidase 9 (MMP9), suppresses metastases. It has been reported that miR-145 exerts its anti metastatic role by directly targeting also the metastatic gene *mucin 1* [54] in breast cancer and VEGF in osteosarcoma cells [55]. Moreover, suppression of Mucin1 by miR-145 causes a reduction of β-catenin as well as the oncogenic Cadherin 11 [54]. Accordingly miR-145, acting as a metastasis suppressor, is stepwise downregulated in normal gastric mucosa, primary gastric cancers and their secondary metastases [53] , and in osteosarcoma in comparison to normal tissues [55].

Several miRNAs such as miR-34a [56], miR-373 and miR-520c [57] and mir-328 [58] have been reported to regulate the cell-surface glycoprotein encoding gene CD44 (cell surface receptor for hyaluronan). Cell lines with high CD44+/CD24− cell numbers are basal/mesenchymal or myoepithelial types and are more invasive than other cell lines [59]. miR-520/373 has been also reported to directly target TGFBR2 and to induce the suppression of Smad-dependent expression of the metastasis-promoting genes parathyroid hormone-

related protein, plasminogen activator inhibitor-1 and angiopoietin-like 4, thus impairing tumor cell invasion, *in vitro* and *in vivo*. Remarkably, decreased expression of miR-520c correlated with lymph node metastasis specifically in ER negative breast tumors [60].

Recently Han M and coworkers [61] demonstrated that miR-21 regulates epithelial-mesenchymal transition phenotype and hypoxia-inducible factor-1α (HIF-1α) expression in sphere forming breast cancer stem cells (CSC). Indeed inhibition of miR-21 by antagomir led to reversal of EMT, down-expression of HIF-1α, as well as suppression of invasion and migration, which indicates a key role of miR-21 in regulating CSC-associated features.

EMT is a profound change in cell phenotype that causes immotile epithelial cells to acquire traits such as motility, invasiveness, anoikis and the ability to adapt to environmental changes to continue to invade successfully. Mesenchymal motility program is characterised by elongated and polarized cell morphology and depends upon ECM proteolysis of the moving cells and from integrin interaction with the extracellular matrix.

Urokinase, a serin protease, activating plasmin triggers a proteolysis cascade that, depending on the physiological environment, participates in extracellular matrix degradation. miR-193a/b overexpression in MDA-MB-231 and MDA-MB-435 breast carcinoma cells significantly reduced its direct target uPA protein amounts and inhibited cell invasion [62]. In an immunodeficient mouse model, miR-193b significantly inhibited the growth and dissemination of xenografted tumors [63]. The expression of miR-193b is downregulated in metastatic breast cancer, and this microRNA is in turn able to upregulate uPA expression and to contribute to the progression of breast cancer. Recently, miR-23b was reported to directly target uPA and c-MET and to decrease migration and proliferation of human hepatocellular carcinoma cells [64]. Plasminogen activation can be regulated also indirectly by mir-17/20 expression, which is usually downregulated in highly invasive breast cancer cell lines and node-positive breast cancer specimens [65]. microRNA17/20 directly repressed IL-8 by targeting its 3' UTR, and inhibited cytokeratin 8 via the cell cycle control protein cyclin D1, a secreted plasminogen activator. Indeed cell-conditioned medium from microRNA17/20-overexpressing non invasive breast cancer cell MCF7 was sufficient to inhibit MDA-MB-231 cell migration and invasion [65]. The invasion-related urokinase receptor is also indirectly regulated by a miRNA. Indeed, oncogenic miR-21, elevated in different tumor types, included colorectal cancer [66] melanoma and breast cancer [67], post-transcriptionally regulates PDCD4, that can suppress invasion and intravasation, at least in part by inhibiting expression of uPAR gene via the transcription factors Sp1/Sp3. Thus, miR-21 is able to enhance cancer cell intravasation, extravasation and metastasis in addition to cell proliferation.

The restoration in hepatocellular carcinoma cells of miR-122 that exerts some of its action via regulation of ADAM17 caused a dramatic reduction of *in vitro* migration, invasion, *in vivo* tumorigenesis, angiogenesis, and local invasion in the liver of nude mice [68]. Under the transcriptional control of HNF1A, HNF3A and HNF3B, miR-122 is specifically repressed in a subset of primary tumors that are characterized by poor prognosis [69].

ADAM9 is directly target by miR-126, which expression is reduced in invasive ductal adenocarcinoma (IDA) and pancreatic cancer cell lines. Re-expression of miR-126 and siRNA-based knockdown of ADAM9 in pancreatic cancer cells resulted in reduced cellular migration, invasion, and induction of epithelial marker E-cadherin [70]. It also directly regulates the adaptor protein Crk that binds to several tyrosine-phosphorylated proteins, inhibiting cell growth by inducing cell cycle arrest in G0/G1 phase, migration and invasion *in vitro* as well as tumorigenicity and metastasis *in vivo* in gastric cancer [71].

Matrix metalloproteinase-2 (MMP2), involved in matrix degradation and involved in angiogenesis, is directly regulated by miR-29b, whose down modulation promotes angiogenesis, invasion, and metastasis of hepatocellular carcinomas [72]. MMP2 was confirmed to be a miR-29b target apart from Mcl-1, COL1A1, and COL4A1 also in prostate cancer cells [73].

miR-29c-targeted genes identified in nasopharyngeal carcinomas (NPC) encode extracellular matrix proteins, including multiple collagens and Laminin γ1, that are associated with tumor cell invasiveness in culture and increased metastasis in animal models and multiple human solid tumors as well as fibrillin SPARC [74]. Interestingly, introduction of miR-29c led to a reduced transcription of these genes in cultured cells, and the down-regulation of mir-29c level in NPC human cancer correlated with increase of target mRNAs, which could facilitate rapid matrix generation and renewal during tumor growth and the acquisition of tumor motility.

It was demonstrated that miR-183 targets ezrin, an intermediate between the plasma membrane and the actin cytoskeleton involved, together with radixin, in epithelial cell morphogenesis and adhesion [75], and may play a central role in the regulation of migration and metastasis in breast cancer [76], osteosarcoma [77] and lung cancer [78]. miR-183 is markedly down-regulated in osteosarcoma cells and tissues compared with matching normal bone tissues and its expression levels significantly correlated with lung metastasis as well as with local recurrence of osteosarcoma [77].

miR-223 is overexpressed in metastatic gastric cancer cells and stimulates non metastatic gastric cancer cells to migrate and to invade. Mechanistically, miR-223, induced by the transcription factor Twist, post transcriptionally downregulates EPB41L3 expression, thought to be involved in tethering the F-actin cytoskeleton to membrane proteins. Another functional downstream target of miR-223 is FBXW7, shown to have important roles in regulating the stability of multiple oncoprotein substrates, including Cyclin E, c-MYC, Notch, c-Jun, and Mcl-1. Overexpression of miR-223 is associated with poor metastasis-free survival in primary gastric carcinomas [79], with lymph node metastasis in gastric cancer, and poorer prognosis in oesophageal squamous cell carcinoma patients [80].

Cells can move also through an "amoeboid invasion" program. This motility style is largely independent from cell-ECM contact and from proteolytic degradation of ECM from MMPs. Furthermore, cell-ECM attachments of amoeboid moving cells are not organized in large focal adhesions but are very diffuse, and much weaker cell-ECM attachments are required,

indeed, amoeboid movement cannot be blocked by inhibition of integrin function. The amoeboid invasion depends from Rho/ROCK expression, and their expression can be regulated also by miRNAs.

miR-10b, the first miRNA described to be pro-metastatic by Ma and colleagues in 2007 [81], inhibits the translation of mRNA encoding HOXD10, increasing the expression of Rho C, and thus leading to tumor cell invasion and metastasis. Ectopic expression of this miRNA endowed non-aggressive human breast cancer cells with the capacity to become invasive, as well as seed distant micrometastases when implanted as xenografts in immunodeficient mice. miR-10b was down-regulated in most breast cancers in comparison with normal mammary tissues, whereas it was highly expressed in about 50% of breast metastatic tumors. Induced by transcription factor Twist, miR-10b function as a metastasis driver in different types of cancer: i.e pancreatic [82], gastric [83] and colorectal [84] cancers.

RhoA, another member of Ras homolog gene family, was described also as a target for miR-155, a Smad4 regulated miR in breast cancer [85].

Mir-31 is able to inhibit multiple steps in the metastatic process: local invasion, one or more early post-intravasation events (intraluminal viability, extravasation efficiency and/or capacity to initially survive in the lung parenchyma), and metastatic colonization. MiR-31 carries out its anti-metastatic function regulating three genes: Rho A, Integrin α5 and Radixin. Via suppression of Rho A, it is able to inhibit *in vitro* invasiveness [86,87]. Notably it also reduces Integrin α5, a key effector of the mesenchymal invasion program, causing concomitant inhibition of both single-cell invasion programs. Controlling expression of Radixin, miR-31 causes anoikis-mediated cell death. In agreement with these data, miR-31 expression has been found to be attenuated in human breast [88-90], prostate [91], ovary [92], stomach [93,94] and bladder cancer [95]. Moreover miR-31 levels in primary human breast tumors were inversely associated with distant metastases [86]. Paradoxically, upregulation of miR-31 in human colorectal [96,97], liver [98] and head-and-neck tumors [99,100], as well as squamous cell carcinomas of the tongue [101] has also been observed [102] .

ROCK1 and ROCK2, the downstream targets of Rho A and Rho C, are regulated by miR-148a [103] and by miR-139 [104], respectively, which all behave as anti-metastatic miRNAs. Overexpression of miR-148a in gastric cancer cells [103] as well as in head and neck cancer cells [105], and of miR-139 in hepatocellular carcinoma cells (HCC) [104], suppressed cell migration and invasion *in vitro* and lung metastasis formation *in vivo*. Accordingly, miR-148a expression was suppressed in gastric cancer compared with their corresponding non tumor tissues, and the downregulated miR-148a was significantly associated with tumor node metastasis and miR-139 expression is reduced in metastatic HCC tumors compared with primary tumors.

In addition, others miRNAs with prominent roles in breast cancer metastasis have been reported.

The c-MYC regulated miR-17-92 cluster, which targets the connective tissue growth factor (CTGF) and the anti-angiogenic adhesive glycoprotein Thrombospondin 1 (TSP1) is shown to be elevated in metastatic breast cancer cells compared with nonmetastatic cells. miRNAs

belonging to this cluster, attenuating also the TGFβ signaling pathway, indirectly shut down clusterin and angiopoietin-like 4 expressions, thereby stimulating angiogenesis and tumor cell growth [106]. Accordingly, blockade of miR-17 is shown to decrease breast cancer cell invasion/migration *in vitro* and metastasis *in vivo* [107]. Furthermore miR-17 and miR-20a were found to be significantly associated with reduced progression free survival in gastrointestinal cancer patients [108] .

The team led by Joan Massague [109] found that miR-335, miR-126, and miR-206 are metastasis-suppressors. Authors performed array-based miRNA profiling in MDA-MB-231 breast cancer cell derivatives (LM2) highly breast cancer cell derivatives highly metastatic to bone and lung, and found a signature of six genes (miR-335, miR-126, miR-206, miR-122a, miR-199a*, and miR-489) whose expression was highly decreased in metastatic cells. Restoring the expression of miR-335, miR-126 or miR-206 in LM2 cells decreased the lung colonizing activity of these cells by more than fivefold. They found that miR-335 suppresses metastasis and migration by targeting the progenitor cell transcription factor SOX4 and TNC messenger RNAs. Consequently, loss of miR-335 leads to the activation of SRY-box containing SOX4 and TNC, which are responsible for the acquisition of metastatic properties. Notably, knockdown of SOX4 and TNC using RNA interference diminished *in vitro* invasive ability and *in vivo* metastatic potential, evidencing that both genes are key effectors of metastasis [110]. miR-126, has recently been described to suppress metastatic endothelial recruitment, metastatic angiogenesis and metastatic colonization through coordinate targeting of IGFBP2, PITPNC1 and MERTK, novel pro-angiogenic genes and biomarkers of human metastasis through the IGFBP2/IGF1/IGF1R and GAS6/MERTK signaling pathways [111]. In addition, low expression of miR-335 or miR-126 in primary tumors from patients was associated with poor distal metastasis-free survival.

IGF1R was identified as a direct target of miR-493 that has been described as a metastasis inhibitor. Indeed, high levels of miR-493 and miR-493(*), but not pri-miR-493, in primary colon cancer were inversely related to the presence of liver metastasis, and attributed to an increase of miR-493 expression during carcinogenesis [112].

Raf kinase inhibitor protein (RKIP) represses breast tumour cell intravasation and bone metastasis through inhibition of MAPK leading to decreased transcription of LIN28 by Myc. Suppression of LIN28 enables enhanced let-7 processing in breast cancer cells. let-7 appears to play a major role in regulating stemness however elevated let-7 expression inhibits HMGA2, a chromatin remodelling protein that activates pro-invasive and pro-metastatic genes, including Snail. LIN28 depletion and let-7 expression suppress bone metastasis, and LIN28 restores bone metastasis in mice bearing RKIP-expressing breast tumour cells [113].

miR-146a is very similar to miR-146b, which is encoded by a different gene, but differs by only two bases and appears to function redundantly in many systems as mediators of inflammatory signaling, influencing differentiation, proliferation and apoptosis. They are pleiotropic regulators of tumorigenesis, as altered expression of both miR-146a/b have been linked with cancer risk, tumor histogenesis and invasive and metastatic capacity in diverse cancers [114]. In fact Hurst and coworkers [27], showed that breast cancer metastasis

suppressor 1 (BRMS1), a protein that regulates expression of multiple genes [115] leading to suppression of metastasis, significantly up-regulates miR-146a and miR-146b in metastatic breast cancer cells. Transduction of miR-146a or miR-146b into MDA-MB-231 inhibited invasion and migration *in vitro*, and suppressed experimental lung metastasis. Bhaumik et al. confirmed that expression of miR-146a/b in MDA-MB-231 cells, impaired invasion and migration capacity by suppressing NF-κB activity [116]. In Table 2 both suppressing and promoting metastasis related miRNAs are summarized.

miRNA	Target
Suppressing miRNAs	
miR-17/20	IL8, Cyclin D1
miR-23b	c-MET, uPA
miR-29b	COL1A1,COL4A1, MMP-2
miR-29c	Laminin γ1, collagens
miR-30a	Snail
miR-31	Integrin α5, Radixin, Rho A
miR-34a	Snail, CD44, c-MET
miR-122	ADAM17
miR-126	ADAM9, Crk, IGFBP2, PTPNC1, MERTK
miR-139	ROCK2
miR-145	Mucin 1, N-cadherin, VEGF
miR-146a,b	NF-κB
miR-148a	ROCK
miR-155	Rho A
miR-183	Ezrin
miR-193a,b	uPA
miR-203	Slug, Snail1
miR-205	ZEB1, ZEB2
miR-335	SOX4, TNC
let-7	HMGA2, RAS
miR-200 family	ZEB1, ZEB2
miR-328	CD44
miR-373/520c	CD44, TGFBR2
miR-493	IGF1R
Promoting miRNAs	
miR-9	E-cadherin
miR-10b	HOXD10
miR-17-92	CTGF, TSP1
miR-21	PDCD4
miR-27	APC
miR-103/107	Dicer
miR-221/222	TRPS1
miR-223	EPB41L3, FBXW7

Table 2. microRNAs and their targets relevant in metastasis.

5. miRNAs as prognostic biomarkers

After early studies indicating the role of microRNA genes in the pathogenesis of human cancer, platforms to assess the global expression of microRNA genes in normal and diseased tissues have been developed. Gene expression profiling has already demonstrated its effectiveness at subtyping various cancers, however miRNA profiles are equally discriminatory and can even be more informative, as changes in their expression can provide insights into the myriad of gene permutations observed in various cancer subtypes: links have indeed been made between misregulated miRNAs and the target genes that are affected, thus unraveling some of the unique gene networks involved [117]. miRNA profiles may identify cancer-specific signatures distinguishing between normal and cancerous tissue [118-121], but they can also discriminate different subtypes of a particular cancer [119,122,123].

To discover microRNAs regulating the critical transition from ductal carcinoma in situ to invasive ductal carcinoma, a key event in breast cancer progression, Volinia et al. [124] performed a microRNA profile on 80 biopsies from invasive ductal carcinoma, 8 from ductal carcinoma in situ, and 6 from normal breast selected from a recently published deep-sequencing dataset [125]. They found that the microRNA profile established for the normal breast to ductal carcinoma in situ transition was largely maintained in the in situ to invasive ductal carcinoma transition. Nevertheless, a nine-microRNA signature that differentiated invasive from in situ carcinoma was identified. Specifically, let-7d, miR-210, and -221 were down-regulated in the in situ and up-regulated in the invasive transition, thus featuring an expression reversal along the cancer progression path. Additionally, they identified microRNAs for overall survival and time to metastasis. Five noncoding genes were associated with both prognostic signatures miR-210, -21, -106b*, -197, and let-7i, with miR-210 the only one also involved in the invasive transition.

Concerning the possibility to use miRNAs as prognostic markers to predict outcome, several groups have successfully addressed this issue [123,126-130] and in particular, concerning involvement of microRNAs in metastatic disease. For example several studies conducted on samples from patient with lung cancer assessed the involvement of metastamiRs: Landi et al [122] analyzed 107 male with early-stage squamous cell lung cancers (SQ) and found 5 miRNAs (miR-25, -34c-5p, -191, let- 7e, miR-34a) whose high expression strongly predicted longer SQ survival [122]. In another study, based on miRNA expression profiling of lung adenocarcinoma and SQ, ten miRNAs (hsa-miR-450b-3p, hsa-miR-29c*, hsa-miR-145*, hsa-miR-148a*, hsa-miR-1, hsa-miR-30d, hsa-miR-187, hsa-miR-218, hsa-miR-708* and hsa-miR-375) associated with brain metastasis were identified including miR-145*, which inhibit cell invasion and metastasis. Two miRNA signatures that are highly predictive of recurrence free survival of 357 stage I NSCLC were also identified, one independent of cancer subtype, the other specific for adenocarcinoma or SQ subtype [131]. From a small cohort of 20 NSCLC patients, Donnem and co-workers [132] in addition to miR differentially expressed between NSCLC tumors and normal control, found 37 miRs up/down regulated in tumors derived from patients with short versus long disease specific survival (DSS) including upregulated miR-31, miR-183, let-7a, miR-193b and downregulated miR-205, miR-378, miR-708 and miR-

29c. A further analysis comparing short versus long DSS patients tumors identified significantly altered angiogenesis-related miRs (miR-21, miR-106a, miR-126, miR-155, miR-182, miR-210 and miR-424) [123], on the basis of a small number of cases, found that the reduced expression of miR-17-5p and -30c in malignant mesothelioma correlated with better survival in patients with the sarcomatoid subtype.

Studies relative to tumors in other body districts have been carried out to determine the involvement of miRs in metastatic disease. Heinzelman et al, analysed miRNA expression of 30 human clear cell renal carcinoma (ccRCC) including 10 non-metastatic tumors, 4 tumors with metastasis after 3 years or later and 4 tumors with primary metastasis. They detected a miRNA signature that distinguishes between metastatic and non-metastatic ccRCC, including miR-451, miR-221, miR-30a, miR-10b and miR-29a. Furthermore, the authors identified a group of 12 miRNAs, such as let-7 family, miR-30c, miR-26a, which are decreased in highly aggressive primary metastatic tumours. They found also correlations between expression levels of specific miRNAs with progression-free survival and overall survival [133].

Veerla et al [95], by miRNA expression analysis of 34 cases of urothelial carcinomas identified 51 miRNAs that discriminated the 3 pathological subtypes Ta, T1 and T2-T3. A score based on the expression levels of the 51 miRNAs, identified muscle invasive tumors with high precision and sensitivity. miRNAs showing high expression in muscle invasive tumors included miR-222 and miR-125b and in Ta tumors miR-10a. Moreover authors identified 2 miRNAs, miR-452 and miR-452*, associated with metastases in the lymph nodes and with a strong prognostic impact on death as endpoint.

353 gastric samples from two independent subsets of patients from Japan were analysed by Ueda et al [119], with the aim to assess the relation between microRNA expression and prognosis of gastric cancer. They found a progression-related signature including miR-125b, miR-199a, and miR-100 as the most important microRNAs involved. Moreover they found that low expression of let-7g and miR-433 and high expression of miR-214 were associated with unfavourable outcome in overall survival independent of clinical covariates, including depth of invasion, lymph-node metastasis, and stage.

6. Conclusion

Although miRs that have been demonstrated to be implicated in the metastatic process might represent a possible therapeutic tool, there have been so far few reported successes in the development of miRNAs for use in therapy. There are two main strategies to target miRNAs expression in cancer. Direct strategies involve the use of oligonucleotides or virus-based constructs to either block the expression of an oncogenic miRNA or to reintroduce a tumor suppressor miRNA lost in cancer. The indirect strategy involves the use of drugs to modulate miRNAs expression by targeting their transcription and their processing. Indeed, even though a number of reports have described the possibility to reintroduce or inhibit microRNAs (reviewed by Iorio and Croce, [134]), there are still many issues that need to be addressed for an effective translation in clinics, as the development of efficient methods of a specific drug delivery, and the accurate prevision of putative unwanted off target effects.

Nevertheless, the results obtained up to date seem quite promising and encouraging, and even though we still have to improve the knowledge in microRNA field to even think of future therapeutic applications, we might be not so far from there.

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