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Functional Roles of microRNAs in Cancer: microRNomes and oncomiRs Connection

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1. Introduction

Cancer is a complex group of diseases characterized by the presence of cells with uncontrolled growth, and high proliferation capacity. The complexity of cancer properties was outlined as the "hallmarks of cancer" a decade ago by Hanahan and Weinberg [1], and it comprises six alterations in cell physiology that dictate malignant growth including: (i) self-sufficiency in growth signals and uncontrolled growth of cells; (ii) insensitivity to anti-growth signals; (iii) evasion of apoptosis; 4 (iv) limitless replicative potential; (v) sustained angiogenesis; and (vi) acquisition of invasive properties to adjacent tissues and organs [1, 2]. These processes are regulated by protein-encoding genes whose expression switches-on or -off during development and in response to cellular environment. Altered versions of the genes (tumor-suppressor genes and proto-oncogenes) which control the normal cellular processes arise from mutations, or expression deregulation in a multistep process resulting in cancer [3]. At the end of the transformation process, the malignant cells acquire growth independence, invasiveness and resistance to senescence and apoptosis. The acquired capabilities of cells to metastasize to other tissues and organs represent the most deadly hallmark of cancer [4-6].

Recently it has been noted that the level of complexity in the mechanisms leading to tumorigenesis has increased as new molecular players in cancer have been identified. Particularly, it has been reported that an abundant class of small non-coding single-stranded RNAs of ~22 nucleotides including microRNAs, and long non-coding RNAs may have relevant roles in cancer. It has been well documented that the expression of microRNAs is strongly deregulated in almost all human malignancies. Functional characterization of these aberrantly expressed microRNAs indicates that they might also function as oncogenes and

tumor suppressors, thus they have been collectively named as "oncomiRs" [5]. Deregulation of microRNAs expression strongly alters key events leading to cancer, including differentiation, proliferation, apoptosis, migration, invasion and metastasis, and chemotherapy resistance. In consequence, the identification of deregulated microRNAs in cancer and their respective targets may provide potential diagnostic and prognostic tumor biomarkers and represent new therapeutic targets for cancer therapy.

2. MicroRNAs biogenesis

MicroRNAs are a class of small non-coding single-stranded RNAs around 21-23 nucleotides length which inhibit gene expression through transcriptional repression and degradation of protein-coding messenger RNAs, in animals, plants and unicellular eukaryotes [7, 8]. The process of microRNAs biosynthesis involves a transcription of hairpin-shaped long transcripts generated by RNA polymerase II (RNA pol II), followed by the endonucleolytic cleavage, mediated by two type III ribonucleases enzymes (RNAse III) known as Drosha (in nuclei) and Dicer (in cytoplasm). The first microRNA (lin-4) was discovered in 1993 in the nematode *Caenorhabditis elegans*. Since it has been estimated the existence of around 17,341 mature microRNAs in 142 species and it has been estimated 1,223 human microRNAs by computational predictions [9, 10]. Analysis of complete genomes sequences from diverse species indicates that most microRNAs genes are located in intergenic non-coding regions, but they are also found within exonic or intronic regions in either sense or antisense orientation and are independently transcribed from their own promoters. The microRNAs localized within introns of protein-encoding or -non-encoding genes (pseudo-genes) have been denominated "mirtrons" [11]. These mirtrons are co-transcribed with their host genes. MicroRNAs genes can be grouped into families by their sequence similarity and function, and they can be localized as single units or grouped in clusters in the genome. It has been estimated that a single microRNA can negatively regulate hundreds or even thousands of target genes indicating that about 30% of human genes could be regulated by microRNAs [12]. However, the functions and cellular targets of most of microRNAs remain to be determined.

The first step in canonical microRNAs biogenesis pathway in animals begins with the transcription of the microRNA gene by the RNA pol II producing a long primary transcript denominated as primary miRNA (pri-miRNA). Clustered microRNAs might be transcribed from a single transcription unit as a polycistronic pri-miRNA. Primary microRNAs contain both 5'-cap structure (7MGpppG) as well as 3'-end polyadenylated tails [13]. After the synthesis of pri-miRNA, the molecule is folded itself into a specific secondary structure of stem-loop that is recognized and cleaved by the microprocessor complex comprised of the RNAse III enzyme Drosha and the DiGeorge syndrome critical region protein 8 (DGCR8). The DGCR8 protein interacts with the pri-miRNA and function as a molecular driver to determine the precise cleavage site. After cleavage of primary microRNA a molecule of 70-100 nucleotides length with a stem-loop structure called precursor microRNA (pre-microRNA) is produced [14, 15]. This post-transcriptional maturation of microRNAs precursors is regulated in response to diverse cellular stimuli. In non-canonical microRNAs

biogenesis pathway, mirtrons are produced without the intervention of the microprocessor complex. Like canonical microRNAs, these ''mirtrons'' are encoded within short stem–loop structures. However, these stem–loops are located within short introns of protein-coding genes, which are released upon pre-mRNA splicing mechanism [16]. The pre-miRNA excised by splicing exhibits a lariat intron form which is subsequently linearized by the debranching enzyme DBR1. Today, it has been described in humans about 13 different mirtrons [17]. These pre-microRNAs are transported to cytoplasm by exportin 5 and Ran-GTP. Pre-miRNAs present a short stem plus a ~2-nt 3' overhang, which is recognized by the nuclear export factor exportin 5 (Figure 1).

Once in the cytoplasm the pre-miRNAs are processed by DICER enzyme (dicing process), another RNAse III enzyme, which together with the dsRNA-binding protein TRBP2 cuts out of the loop and generates an imperfect double stranded RNA formed by the guide (miRNA) and transient strand (miRNA*) which is degraded by AGO2. However, it has been recently established that miRNA* strand is also functional. Subsequently, TRBP2 recruits the protein Argonaut 2 (AGO2) to the complex microRNA/DICER forming the silencing complex induced by RNA (RISC), which preferentially includes the mature single-stranded miRNA molecule and AGO proteins (AGO2-4), acting as guiding molecules to deliver the complex to target mRNA[18]. The mature microRNA then hybridizes to nearly complementary sites in the 3' untranslated region (3'-UTR) of mRNA targets. Negative gene expression regulation mediated by microRNAs depends on the degree of complementarity between the microRNA and its target mRNA. Translational repression of transcripts is driven when microRNA binds to target with imperfect complementarity. This imperfect miRNA:miRNA interaction means that a single microRNA can potentially target tens to hundreds of mRNAs. When microRNAs binds to its mRNA targets with a high complementarity, the degradation of the messenger is induced [19]. Notably, the microRNAs mediated-decay of mRNA targets is initiated by shortening of poly(A$^+$) transcripts by the canonical deadenylation machinery that includes the CAF1 deadenylase.

Importantly, translation repression and/or degradation of mRNA targets by microRNAs occurs in cytoplasmic foci denoted as mRNA processing bodies (P-bodies) which are enriched in mRNA decay factors and pools of stored messenger ribonucleoproteins [20]. P-bodies are mRNA processing centers within which non-translating transcripts are sorted and either silenced or degraded. Although the protein inventory of P-bodies has not been defined in detail, around 25 different factors have been detected within these cytoplasmic foci. The P-bodies observed in yeast, insect, nematode and mammalian cells have critical roles in mRNA degradation, mRNA storage, mRNA surveillance and RNA-based gene silencing mechanisms [21]. Moreover, it has been demonstrated that P-bodies have high concentration of target transcripts and the AGO2, DICER, and GW182 proteins involved in RNA interference by microRNAs and small interfering RNAs [22, 23].

Although the main function of microRNAs is the gene regulation at the post-transcriptional level, it has been observed that microRNAs can also activate or repress gene expression at

transcriptional level. Recently, it has been evidenced that mature microRNAs may also be localized in nucleus, through a specific hexanucleotide (AGUGUU) sequence which acts as a transferable nuclear localization element [24]. An example of this is the positive regulatory effect on transcriptional level of mir-122 in liver cells [25]. Moreover, it has been shown that vesicles of endocytic origin known as exosomes may contain both mRNA and microRNAs, which can be delivered to adjacent cells, and can be functional therein. These novel RNA molecules are known as exosomal shuttle RNAs as they mediate exchange of microRNAs with other cells which represents a exciting mechanism of genetic exchange [26].
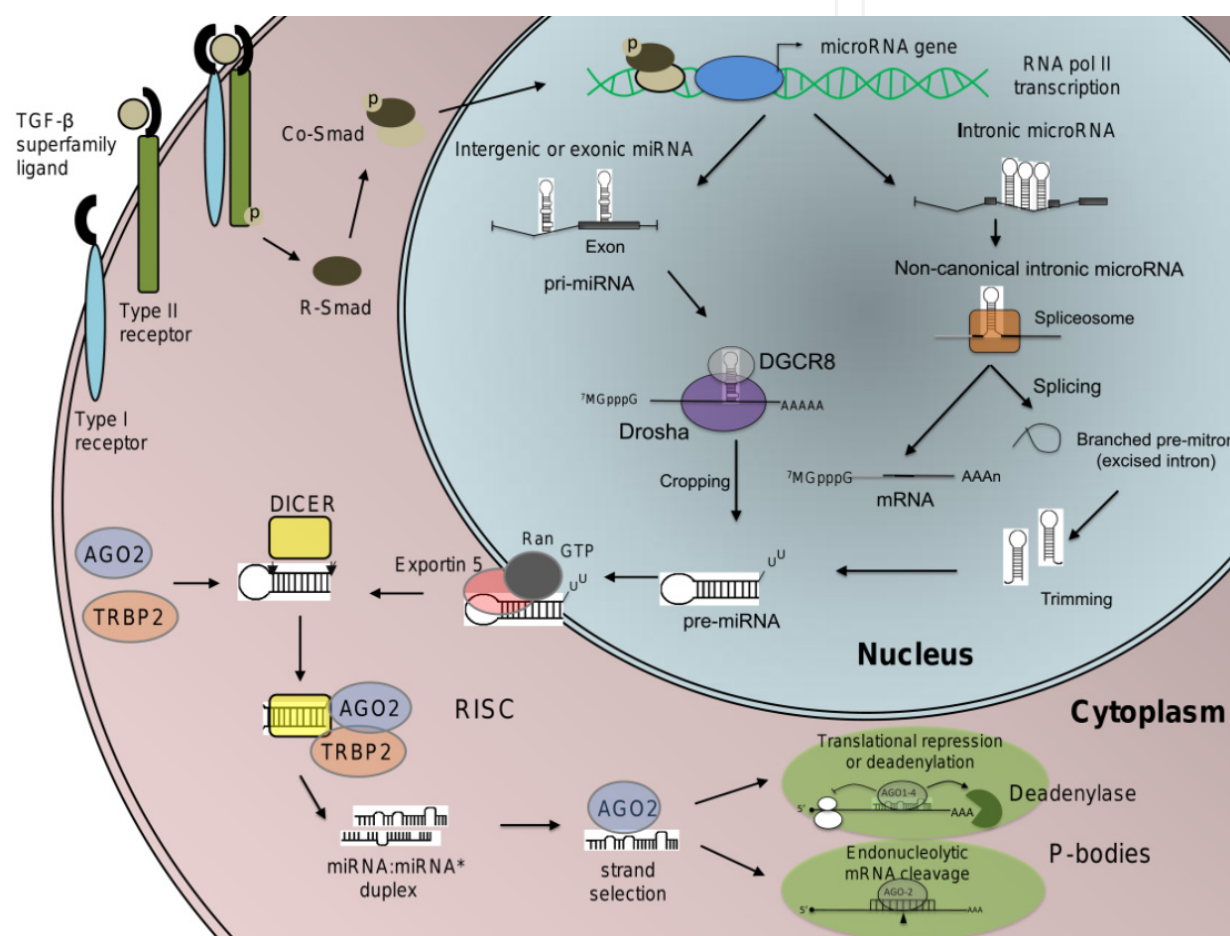
Figure 1. MicroRNAs biogenesis in mammals. The activation of microRNAs transcription by transforming growth factor beta (TGF-β) ligands and sequential phosphorylation of type II receptors activating type I receptors, which in turn activate R-Smads receptors and Smad signaling is depicted. MicroRNAs are transcribed by RNA Pol II in nuclei to generate the primary microRNA (pri-miRNA). These large non-coding RNAs are processed by the endoribonuclease Drosha and DGCR8 proteins (microprocessor complex) to produce the precursor microRNA (pre-miRNA). Mirtrons are alternatively produced during splicing of introns of messenger RNAs. Then, pre-miRNA is exported to cytoplasm by exportin 5/Ran-GTP system. In cytosol, pre-miRNA is processed by Dicer and TRBP2 complex to generate the mature microRNA which then binds to Argonaute family of proteins (AGO1-4) forming the RNA induced silencing complex (RISC). Finally, microRNAs may either inhibit the translation of mRNA or promote mRNA degradation. These events occur in discrete cytoplasmic structures denoted as P-bodies.

2.1. Regulation of microRNAs biogenesis

The biosynthesis of the microRNAs can be regulated at different levels. It has been defined that microRNAs are transcribed by RNA pol II. Primary transcripts present the same characteristics of the mRNAs, including 7-methylguanylate cap structure at the 5'-end, and poly (A) tail at the 3'-end. The majority of DNA-binding elements and transcription factors binding sites in microRNAs promoters largely overlap with those that control protein-coding genes, such as *c-myc* or *p53*. On the other hand, transcription of primary miRNA transcripts can be dynamically regulated in response to growth factor stimulation, including platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β) and Smad [27]. Epigenetic control of microRNAs expression is another regulation level which includes DNA methylation and histones modifications. For example, the expression of miRNA-127 is reduced due to promoter hypermethylation in bladder cancer [28]. Another example is the regulation of miRNA-1 by inhibition of histone deacetylase (HDAC) enzyme in breast and lung cancers [29]. In addition, it has been reported that mutations in genes coding for Drosha and Dicer occurs in a variety of cancers.

3. MicroRNAs and cancer: OncomiRs

Deregulation of microRNAs expression is common in all types of human cancer. Early studies showed a differential microRNAs expression profiles between tumors and normal tissues [30-32]. Moreover, alterations in microRNAs expression correlate with severity of disease, as they regulate key transcripts involved in initiation and progression of tumors. However, these observations does not imply that deregulated microRNAs are directly involved in tumor development and progression, as they could be indirectly altered by the genetic and epigenomic changes that arise during carcinogenesis. MicroRNAs can act as truly oncogenes or tumor suppressors to inhibit or exacerbate the expression of cancer-related target genes, and to promote or suppress tumorigenesis, thus they have been denominated as oncomiRs [33]. Those microRNAs whose expression is increased in tumors may be considered as oncogenes, as they usually promote tumor development by inhibiting tumor suppressor genes and/or genes that control cell cycle, cell differentiation and apoptosis [34]. Contrarily, when the expression of microRNAs is diminished in cancer cells, they are considered as tumor suppressor genes. Tumor suppressor microRNAs usually prevent tumor development by inhibiting oncogenes and/or genes that control cell differentiation or apoptosis [33]. Deregulation of microRNAs expression frequently arises from genetic or epigenetic alterations, represented by deletions, amplifications, point mutations and aberrant DNA methylation events. Remarkably, about half of the human microRNAs are located within fragile regions of chromosomes, which are domains of the genome that are frequently lost in various human cancers [35].

Of clinical interest, the large high-throughput studies in patients revealed that microRNAs profiling has the potential to classify tumors and predict patient outcome with high accuracy [31, 32, 36]. For example, it was shown that high levels of miR-155 and low let-7a-2 expression correlate with poor survival in patients with lung adenocarcinoma [37]. In

addition, microRNA profiling in lung cancer identified five microRNAs important for prognosis. Results showed that high levels of miR-221 and let-7a appeared to be protective, while high levels of miR-137, miR-372, and miR-182 were correlated with worse clinical outcome [38]. Another study in colorectal cancer showed that high miR-21 expression was associated with poor survival and poor therapeutic outcome [39]. Moreover, components of the microRNA-based gene silencing machinery have also been implicated in tumorigenesis. Reduced expression of Dicer has been shown to be down-regulated in lung cancer and associated with poor prognosis [40]. These findings are in agreement with previous reports of Dicer loss in some tumors. In addition, other reports showed that low expression levels of Drosha were significantly associated with advanced tumor stage in ovarian cancer [41]. Other components of the microRNA machinery that have been implicated in cancer include Argonaute family members Ago1, Ago3, and Ago4 which cluster on the 1p34-35 chromosomal region, that is often lost in in human cancers such as Wilms tumors, neuroblastoma and breast, liver and colon carcinomas [42]. In summary, emerging evidence suggests that oncomiRs play important roles in human cancers. Several microRNAs may be directly involved in cancer development by controlling cell differentiation and apoptosis, while others may be involved in cancer by targeting cancer oncogenes and/or tumor suppressors. Next, we will discuss the roles of microRNAs in metastasis, the more deadly hallmark in cancer.

4. MetastamiRs: microRNAs driving invasion and metastasis

4.1. Mechanisms of invasion and metastasis

The ability of cancer cells to metastasize is a hallmark of malignant tumors. Metastatic development from primary tumor to a secondary organ or tissue should be successfully complete in multiple sequential steps that include spreading of tumoral cells from primary tumor, enhanced motility, intravasation, extravasation and colonization in a secondary site to form a distant tumor [43, 44]. Each step in this complex cellular process represents a physiological barrier that must be overcome by the tumor cells for successful metastasis [45]. These events are regulated by genetic and epigenetic programs that are acquired during tumor progression [46]. However, it remains unclear if oncogenic transformation is sufficient for metastatic competence. The long latency period of certain tumor types suggests a further evolution of malignant cells in the microenvironments of particular organs [47]. Metastasis initiation genes allow cancer cells to invade the surrounding tissues, attract a supportive stroma and facilitate cellular dispersion and infiltration in distant tissues [6]. These genes participate in the regulation of motility, epithelial-mesenchymal transition (EMT), adhesion and proteolysis. They determine tumor cells interactions with other cells and with the extracellular matrix, and activate migration, angiogenesis and survival [48]. These genes codified for transcription factors, growth factors receptors, protein kinases and importantly microRNAs [49, 50]. Tumor cells interactions with the extracellular matrix are mediated by integrins which trigger invasion and spread. These proteins promote invasion and proliferation and they determine whether cells migrate and proliferate in response to cytokines and growth factors [51]. Metastatic cells loss their adherent junctions, which, in

epithelial cells, are constituted primarily by E-cadherin. E-cadherin is replaced by N-cadherin, which plays an important role in invasion by regulating fibroblast growth factor receptor (FGFR) function [52]. This process, known as the cadherin switch, is associated with EMT, allowing the conversion of epithelial cells to motile fibroblast-like cells that express mesenchymal rather than epithelial cell markers [53]. The communication of epitheliai cell with their microenvironment is regulated by E-cadherin-mediated cell-cell interaction and β1-integrin-mediated adhesion to the basement membrane (BM), which is the first barrier to invasion by carcinoma cells. Other proteins involved in the metastatic process include the matrix metalloproteinases (MMP), COX2 and cytokines. MMP can degrade the components of the BM such as collagen IV. Importantly, MMP promotes angiogenesis, one of the prerequisites for metastatic tumor growth by degradation of the fibrin matrix that surrounds newly formed blood vessels, facilitating endothelial cell penetration of tumor tissues [54].

In the "omics" era, the genomic profiling, second-generation sequencing, proteomics and other global level analytical techniques have dramatically accelerated the efforts to comprehensively characterize metastatic tumour cells and to understand their natural history of evolution from primary tumors [55]. Discovered gene signatures in metastatic tumors have been applied to identify functional drivers of metastasis. Comparing the expression profiles of highly metastatic cells with their weakly metastatic counterparts from an isogenic background allowed for the identification of metastasis-promoting [56-58] and metastasis-suppressing genes [59-61]. In addition, gain-of-function or loss-of-function genomic screens, cross-species integrated genomic analyses and computational reanalysis of genomic profiling data have also led to the identification of functional mediators of metastasis with direct clinical relevance [61]. However, despite great advancements in the knowledge of metastasis biology, the molecular mechanisms are still not completely understood. Remarkably, a regulatory role for microRNAs in metastasis has been established, thus they have been denominated metastamiRs, as they have pro- and anti-metastatic effects [62]. The term metastamiRs was recently introduced by Welch and colleagues to refer to those regulatory microRNAs which promote or suppress various steps in migration and metastasis of cancer cells [63]. It seems that these metastamiRs regulate key steps in the metastatic program and processes, such as EMT and angiogenesis. Most commonly, metastamiRs promoting cell migration and invasion have been described [62]. Next, we will review some of the identified microRNAs with a relevant role in metastasis in   human cancers.

4.2. MetastamiRs in prostate cancer

Prostate cancer is the second more lethal cancer type in men in America [64]. Once it has progressed to metastasis (manly to bone) the disease is currently incurable, since metastatic cells are highly resistant to conventional therapies. The involvement of microRNAs in human prostate cancer has been well documented and some aberrantly expressed microRNAs with critical roles in the progression and metastasis of prostate cancer have been discovered [65]. For example, miR-21 expression levels significantly correlate with advanced clinical stage, metastasis and poor prognosis in prostate cancer. In these studies it has been

evidenced that miR-21 targets myristoylated alanine rich protein kinase c substrate (MARCKS), which is involved in cellular processes, such as cell adhesion and cell motility through regulation of the actin cytoskeleton [66]. In another study, Watahiki and coworkers discovered differentially expressed known and novel microRNAs from a transplantable metastatic compared with non-metastatic prostate cancer xenograft line, both derived via subrenal capsule grafting, and from one patient's primary cancer tissue [67]. These microRNAs seem to have specific roles in the metastasis of prostate cancer. In another report, Gandellini and coworkers showed that miR-205 is overexpressed in normal prostate tissue and RWPE-1 cells, whereas it was almost undetectable in both androgen-dependent and androgen-independent prostate cancer cells [68]. Authors showed that overexpression of miR-205 in prostate cancer cells promotes up-regulation of E-cadherin and reduction of cell locomotion and invasion, suggesting a relation with EMT. Peng and colleagues reported that the expression of five microRNAs (miRs-508-5p, -145, -143, -33a and -100) was significantly decreased in bone metastasis when compared with primary tumor prostate [69]. Notably, miRs-143 and -145, were up-regulated, and they were able to repress migration and invasion *in vitro*, tumor development and bone invasion *in vivo*, as well as EMT of PC-3 derived from metastatic cells. Since the principal problem arising from prostate cancer is its propensity to metastasize to bone, these findings could be important for the understanding of organ specific metastasis in this neoplasia [69].

4.3. MetastamiRs in colorectal cancer

Colorectal cancer is the third most common malignant disease and the fourth leading cause of cancer-related deaths worldwide. Metastases have occurred in about 25% of patients at the time of diagnosis, and an additional 40% to 50% develop secondary metastases during the course of their disease after diagnosis. Currently about 100 miRNAs have been implicated in colorectal cancer [70]. The most up-regulated miRNAs are miR-21, miR 17-92 cluster, miR-135a/b, miR-471 and miR-675, whereas miR-143, miR-14, let-7 and miR-101 showed a decreased expression in colorectal cancer. The main targets of these miRNAs include transcription factors like c-MYC, STAT, OCT4, SOX, E2F1, ZEB1, ZEB2, NFIB and some kinases, such as ERK and YES1, and proteins involved in matrix metalloproteinases regulation like RECK and TIMP3 that functions as metastasis suppressors [71]. It has been reported that up-regulation of miR-21 in colorectal cancer cells increases their migratory and invasive abilities, through regulation of RECK and TIMP3 genes., Dews and coworkers used a mouse model of colon cancer to demonstrate that the angiogenic activity of c-MYC is due at least in part to downstream activation of the miR-17-92 cluster. Authors showed that vascularization of tumors can be induced by expression of either c-MYC or the miR-17-92 cluster [72].

4.4. MetastamiRs in breast cancer

Because of the availability of robust metastasis models, the vast majority of these metastamirs have been identified in breast and/or mammary tumor cell lines [62]. Ma and coworkers from Robert Weinberg's group evidenced that up-regulation of miR-10b

suppressed homeobox D10 (HOXD10) expression, allowing the activation of the pro-metastatic gene RHOC and initiation of breast cancer invasion and metastasis [73]. They showed that miR-10b was overexpressed in metastatic MDA-MB-231 cell line, in comparison with tumorigenic non-metastatic MCF7 cells. Interestingly, the ectopic expression of miR-10b results in increased migration and invasion properties in two different human breast cell lines. In contrast, silencing of miR-10b using antisense inhibitor oligonucleotides led to a 10-fold reduction of the invasive properties from transfected cells. Importantly, overexpression of miR-10b in non-metastatic tumorigenic cell lines promoted robust invasion, and lung distant micro-metastases *in vivo*. Moreover, Ma and coworkers evidenced that TWIST1 [74], a metastasis promoting transcription factor specifically binds to the putative promoter of *mir-10b* gene activating its expression. This induces the inhibition of homeobox HOXD10 transcription [75], leading to an increased expression of the pro-metastatic gene RHOC, a signaling GTPase-protein involved in metastasis. Importantly, it has been described that HOXD10 expression is lost in breast tumors [76]. Finally, Ma and coworkers showed that silencing of RHOC by small interfering RNAs caused repression of miR-10b induced cell migration and invasion. In another outstanding study it was reported that systemic therapeutic silencing of miR-10b in tumor-bearing mice significantly suppressed breast cancer metastasis and increased the levels of its target HOXD10 [77]. In another study it was established that miR-373 and miR-520c promote tumor invasion and metastasis by regulating the cell-surface glycoprotein encoding gene CD44 (cell surface receptor for hyaluronan) [78]. Huang and coworkers from Agami's group set up a genetic screen using the non-metastatic MCF7 cell line, and found that miR-373 and miR-520c stimulated cell migration and invasion both *in vitro* and *in vivo*. Interestingly, authors evidenced that miR-373 and miR-520c "seed" sequences were similar and both CD44 target mRNA. Moreover, enhanced expression of a CD44 gene that was unresponsive to miR-373/miR-520c, inhibited the migratory activity of MCF7 cells overexpressing miR-373 and miR-520c.

The team led by Joan Massague performed an array-based miRNA profiling in MDA-MB-231 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 [79]. 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. Interestingly, miR-126 restoration reduced overall tumor growth and proliferation, whereas miR-335 inhibited metastatic cell invasion. In addition, low expression of miR-335 or miR-126 in primary tumors from patients was associated with poor distal metastasis-free survival. In addition they profiled LM2 cells overexpressing miR-335 and identified 756 genes whose expression was decreased including genes previously implicated in extracellular matrix and cytoskeleton control (type 1 collagen COL1A1) and signal transduction (receptor-type tyrosine protein phosphatase PTPRN2, c-Mer tyrosine kinase (MERTK) 21 and phospholipase PLCB1), as well as in cell migration, such as the tenascin C (TNC), an extracellular matrix glycoprotein of stem cells niches [80] and the SRY-box containing transcription factor SOX4. 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.

It has been reported that miR-146a and b inhibited invasion and migration of breast cancer cells by down-regulating NFκB through IRAK1 and TRAF6 targeting [81]. Both miR-146a and b suppressed metastasis through targeting of EGF receptor and ROCK1 which are involved in promoting invasion and metastasis. In another study, Hurst and coworkers showed that breast cancer metastasis suppressor 1 (BRMS1), a protein that regulates expression of multiple genes leading to suppression of metastasis, significantly up-regulates miR-146a and miR-146b in metastatic breast cancer cells. Moreover, transduction of miR-146a or miR-146b into MDA-MB-231 down-regulated expression of epidermal growth factor receptor, inhibited invasion and migration *in vitro*, and suppressed experimental lung metastasis [82].

In a seminal paper, it was reported that miR-31 inhibited multiple steps of metastasis including invasion, anoikis, and colonization leading to almost a complete reduction in lung metastasis [83]. Clinically, miR-31 levels were lower in breast cancer patients with metastasis. In another study, it was reported that suppression of miR-21 in metastatic MDA-MB-231 breast cancer cells significantly reduced invasion and lung metastasis, by targeting programmed cell death 4 (PDCD4) and maspin, which have been involved in invasion and metastasis. Li and coworkers reported that down-regulation of miR-193b contributes to enhance urokinase-type plasminogen activator expression and tumor progression and invasion in human breast cancer [84]. In other study, it was evidenced that overexpression of miR-200, which promotes a mesenchymal to epithelial cell transition by inhibiting Zeb2 expression, unexpectedly enhances macroscopic metastases in mouse breast cancer cell lines [85]. Vetter and coworkers showed that miR-661 expression in MCF7 breast cancer cells conditionally overexpressing the EMT master regulator SNAI1, contributes to breast cancer cell invasion by targeting cell-cell adhesion Nectin-1 and the lipid transferase StarD10 messengers [86].

5. MicroRNAs and angiogenesis

5.1. Regulation of angiogenesis

The term angiogenesis refers to the growth of new blood vessels from pre-existing vessels. It normally occurs during embryonic development, wound healing, and the menstruation cycle. During angiogenesis, quiescent endothelial cells are activated by angiogenic factors and start to migrate, proliferate and organize themselves in tubular structures [87]. Angiogenesis is a physiological process during development, and plays essential roles in the recovery of blood flow in ischemic tissues. Unregulated angiogenesis is seen in pathological conditions, such as cancer and is a fundamental step in tumor growth. During tumor growth, angiogenesis is required for proper nourishment and removal of metabolic wastes from tumor sites. Inhibition of tumor angiogenesis leads to repression of tumor growth and has been identified as a potential therapeutic strategy.

Angiogenesis is induced by hypoxia as a result of the expression of pro-angiogenic factors through hypoxia-inducible factor-H1 (HIF-1). HIF-1 is the major oxygen homeostasis regulator. It has a key role as transcriptional regulator, orchestrating the expression of a wide variety of genes thought to be critical for adaptation to low oxygen. Under normoxic conditions, HIF-1 is rapidly degraded by the proteasome. However, under hypoxic conditions, HIF-1 is stabilized and activates a highly complex transcription program, comprising hundreds of genes that regulate processes such as angiogenesis (vascular endothelial growth factor (VEGF), endothelial growth factor receptor 1, plasminogen inhibitor 1), glucose metabolism (lactate dehydrogenase A, aldolase A and C, and phosphofructo-kinase L), survival and death (BNIP3, p21, Nip-3 like protein) [88-90]. When pro-angiogenic factors are in excess in comparison with of anti-angiogenic factors, the switch to an angiogenic phenotype can occurs.

6. MicroRNAs and hypoxia

Hypoxia has recently been shown to induce the expression of a number of microRNAs, which have been termed "hypoxamirs" [89, 94]. Members of this group seem to affect apoptotic signaling in a hypoxic environment and are also predicted to target genes of critical importance for tumor biology. Interestingly, most hypoxia-induced microRNAs are also overexpressed in human cancers, suggesting their role in tumorigenesis [92, 94]. Using miRNA expression arrays different hypoxia-regulated microRNAs (HRMs) were determined to be induced in response to hypoxia in breast and colon cancer cells. These HRM were miR-21, miR-23a, miR-23b, miR-24, miR-26a, miR-26b, miR-27a, miR-30b, miR-93, miR-103, miR-106a, miR-107, miR-125b, miR-181a, miR-181b, miR-181c, miR-192, miR-195, miR-210 and miR-213. *In silico* analysis revealed a highly complex spectrum of candidate targets, including genes involved in proliferation, apoptosis, DNA repair, chromatin remodeling, metabolism, and migration. For example, component of the apoptotic machinery were found to be potentially targeted by HRMs: BID (miR-23), BIM (miR-24); CASP3 (miR-30), CASP 7 (miR-23), APAF1 (miR-27), BAK1 (miR-26), Bnip3L (miR-23) [91]. Recently experimentally data confirmed an important regulatory role in HIF-1 for miR-210, 26 and 181 hypoxia-induced microRNAs [91, 92]

In addition to the microRNAs that respond to hypoxia by up-regulation of their expression, the following microRNAs were identified as downregulated in hypoxic cells: miR-122a, miR-565, miR-195, miR-30e-5p, miR-374, 19a, miR-101, miR-424, miR-29b, miR-186, miR-141, miR-320, miR-422b, and miR-197 in SCC cells; miR-15b, miR-16, miR-20a, miR-20b, 30b and miR-224 in CNE cells, and miR-424 in trophoblasts [93]. In addition it has been reported that several microRNAs, including miR-16, miR-20a, miR-20b and miR-320, control expression of VEGF[92, 93].

6.1. Relationships among HIFs and hypoxamirs

The response to hypoxia generates hypoxamirs that can be grouped into three clusters (Table 1):

a. **Hypoxamirs induced by HIF**: Among the HIF-dependent hypoxamirs are miR-210 and miR-373. The most robustly induced hypoxamir, miR-210, is induced by HIF-1α and suppresses expression of the cell-cycle regulator E2F transcription factor 3 (E2F3), the receptor tyrosine kinase ligand ephrin A3, and the DNA repair protein RAD52. In addition, recently it was shown that miR-210 has an important role in suppression of mitochondrial metabolism in hypoxic states by decreasing expression of the iron-sulfur cluster assembly proteins ISCU1/2, thereby limiting cytochrome assembly and ROS generation from inefficient mitochondrial electron transport under low oxygen tensions [94].

b. **Hypoxamirs that affect HIF:** These hypoxamirs are induced by hypoxia and have an effect on HIF expression [94]. Three hypoxamirs have been shown to affect HIF expression: miR20b, miR-199a, and, most recently, miR-424. The miR-20b targets HIF-1α and suppresses its expression in MCF-7 breast cancer cells, and downregulation of miR-199a derepresses HIF-1α in cardiomyocytes. miR-424 regulates HIF-α isoforms in endothelial cells by targeting cullin 2 (CUL2), the scaffolding protein on which the ubiquitin ligase system assembles, thereby stabilizing HIF-α isoforms by impairing their prolyl hydroxylation [94].

c. **MicroRNAs that affect HIF independently of hypoxia.** At least four microRNAs have been shown to influence HIF expression independently of hypoxia. Induced by p53, miR-107 decreases the expression of HIF-β. Induced by c-MYC, the miR17-92 cluster suppresses the expression of HIF-1α. Suppressed by hepatocyte growth factor, miR-519c suppresses the expression of HIF-1α. In contrast, miR-31, by decreasing expression of the HIF regulatory factor factor-inhibiting HIF (FIH), increases the expression of HIF-1α [94].

Hypoxamirs induced by HIF	Hypoxamirs that affect HIF	MicroRNAs that affect HIF independent of hypoxia
miR-210	miR-20b	miR-107
miR-373	miR-199a	miR-17-92 cluster
	miR-424	miR-31
		miR-519c

Based on [94].

Table 1. MicroRNAs regulated by hypoxia

6.2. Transcriptional regulation of hypoxamirs

The expression of microRNAs requires the basic transcription machinery used for protein-encoding genes transcription. Most of the microRNAs transcription depends on RNA polymerase II. Such resemblances hinted to the possibility that microRNA induction/repression could also be controlled by transcription factors. Delineating the promoter regions of microRNAs is a necessary step for an expanded understanding of microRNA expression control. The main challenge comes from the fact that only few microRNA promoters have been identified experimentally [95].   Kulshereshtha and

coworkers analyzed a set of promoters for all the predicted microRNAs in human genome; they predicted HIF-binding sites by position weight matrix approach. The results showed that approximately 6% of the human microRNAs exhibit significantly conserved HIF sites, which could reflect their functional importance. Additional candidate sites for Oct-C, AP2, PPAR γ and E2F transcription factors were also identified in the miR-210 promoter.[96]. These sequences could potentially regulate its expression as part of the hypoxia response. Finally, several studies have shown that microRNA biogenesis machinery is not altered in response to hypoxia. Expression of microRNA processing proteins like Ago2, Drosha, Exp5, Dicer and DP103 does not suffer any expression changes during hypoxia. Additionally Dicer impairs angiogenesis *in vitro* and *in vivo* [97].

6.3. Hypoxamirs expression and cellular context

In the case of hypoxia-regulated microRNAs, *in silico* searches reveal a highly complex spectrum of candidate targets, including genes involved in proliferation, apoptosis, DNA repair, chromatin remodeling, metabolism and migration. One set of targets are cell death regulators, given the importance of this process in a stressful environment, such as hypoxia. Using PicTar, Target-Scan and MirBase prediction programs, a number of core component genes of the apoptotic machinery were found to be potentially targeted by hypoxamirs:: BID (miR-23), BIM (miR-24); CASP3 (miR-30), CASP 7 (miR-23), APAF1 (miR-27), BAK1 (miR-26), Bnip3L (miR-23). Additionally, Bcl2 is also an experimentally confirmed target of miR15 and 16, which were found to respond to hypoxia by down-regulation, at least in CNE cells.

Another process known to be affected by hypoxia is proliferation, since many cell types undergo cell cycle slowdown or arrest during oxygen deprivation. A plethora of cell cycle genes are identified as putative HRMs targets, such as: cdc25A (miR-21, miR-103/107), cyclin D2 (miR-26, miR-103/107), cyclin E1 (miR-26), cyclin H (miR-23), cdk6 (miR-26, miR-103/107) [92]. An additional gene of relevance for this subject is VEGF for which a group of regulatory microRNAs have been identified, including miR-16, miR-20a, miR-20b, let-7b, miR-17-5p, miR-27a, miR-106a, miR-106b, miR-107, miR-193a, miR-210, and miR-320. Interestingly, most of these microRNAs have been identified by at least one of the recent studies as responsive to hypoxia, either by induction or by repression, which could lead to an extra layer of complexity in the angiogenic response. Targeting microRNAs involved in hypoxia control could be applied in clinical oncology, as the majority microRNA identified are overexpressed in some tumor subtypes, suggesting that hypoxia represents a contributing element for microRNA alterations in cancer. Moreover, manipulation of select microRNAs could synergize with conventional therapies.

7. Conclusions

The recent discovery of the role of microRNAs as tumor-suppressor genes or oncogenes has added an additional level of complexity to the mechanisms leading to tumorigenesis. In

particular, the review presented here evidences that metastamiRs have emerged as new molecular players that regulate growth, angiogenesis, invasion, and metastasis events in cancer. Understanding how metastamiRs are involved in regulating tumor invasion and metastasis process will provide a promising strategy for the identification of molecular markers of progression and prognosis, for response to chemotherapy, early biomarkers of aggressive tumors, and the development of new metastamiRs-based treatments. In addition, we highlight the prominent roles of hypoxamirs in cancer. Targeting microRNAs involved in hypoxia control could be applied in clinical oncology. However, further investigations about the role of microRNAs in cancer are required in order to use them as targets for therapy, prognosis and diagnosis in the near future.

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