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J Mol Med (2014) 92:321–336
DOI 10.1007/s00109-014-1129-2
JMolMed
REVIEW

MicroRNAs: master regulators of drug resistance, stemness, and metastasis

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Received: 4 December 2013 / Revised: 21 January 2014 / Accepted: 23 January 2014 / Published online: 9 February 2014
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Abstract MicroRNAs (miRNAs) are 20–22 nucleotides long small non-coding RNAs that regulate gene expression post-transcriptionally. Last decade has witnessed emerging evidences of active roles of miRNAs in tumor development, progression, metastasis, and drug resistance. Many factors contribute to their dysregulation in cancer, such as chromosomal aberrations, differential methylation of their own or host genes' promoters and alterations in miRNA biogenesis pathways. miRNAs have been shown to act as tumor suppressors or oncogenes depending on the targets they regulate and the tissue where they are expressed. Because miRNAs can regulate dozens of genes simultaneously and they can function as tumor suppressors or oncogenes, they have been proposed as promising targets for cancer therapy. In this review, we focus on the role of miRNAs in driving drug resistance and metastasis which are associated with stem cell properties of cancer cells. Furthermore, we discuss systems biology approaches to combine experimental and computational methods to study effects of miRNAs on gene or protein networks regulating these processes. Finally, we describe methods to target oncogenic or replace tumor suppressor miRNAs and current delivery strategies to sensitize refractory cells and to prevent metastasis. A holistic understanding of miRNAs' functions in drug resistance and metastasis, which are major causes of cancer-related deaths, and the development of novel strategies to target them efficiently will pave the way towards better translation of miRNAs into clinics and management of cancer therapy.

Keywords MicroRNAs · Drug resistance · Stemness · Epithelial–mesenchymal transition · Metastasis · miRNA–protein interaction networks · Systems biomedicine

Electronic supplementary material The online version of this article (doi:10.1007/s00109-014-1129-2) contains supplementary material, which is available to authorized users.

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Introduction

miRNAs are a large family of small regulatory RNAs, acting mostly in post-transcriptional gene silencing. They are 20–22 nucleotides long and recognize their target mRNAs by complementary base pairing. They control gene expression by mRNA cleavage, mRNA destabilization, or inhibition of translation [1]. Almost half of miRNAs reside in clusters and transcribed as polycistronic precursor miRNAs [2]. Other miRNAs, located in intergenic regions, are transcribed by their own promoters, and those present in intronic regions are likely under the control of the host genes' promoters [3]. Currently, it has been reported that there are around 2,600 unique mature miRNAs in human (miRBase version 20) [4]. Most miRNAs are transcribed by RNA polymerase II as primary transcripts (pri-miRNAs), usually several kilobases long, which fold into hairpin structures containing imperfectly base-paired stem–loop structures [5]. RNase III endonuclease Drosha then cleaves primary miRNAs (pri-miRNAs) into ~70 nt long precursor miRNAs (pre-miRNAs), which are later transported to cytoplasm by RanGTP-dependent dsRNA-binding protein exportin-5 (XPO5) [6]. In cytoplasm, RNase III endonuclease Dicer cleaves pre-miRNAs into mature

miRNAs which are loaded to RNA-induced silencing complexes. Along with Argonaute (Ago) proteins (mainly Ago1 and Ago2 in mammals) of the complex, miRNAs downregulate gene expression by binding to target mRNAs. Although miRNA binding sites have also been found in 5′-UTR [7] and coding sequence [8] of mRNAs, they preferentially interact with seed-matching sequences in the 3′-UTR of mRNA. One miRNA can downregulate multiple genes due to the short sequence required for mRNA recognition, which is known as the "seed region" spanning between the 2nd and the 7th (or 8th) nucleotide of mature miRNAs. Taking both direct and indirect regulations together, it is not rare that a single miRNA can regulate the expression of tens or hundreds of genes.

Considering the enormous regulatory potential of miRNAs, it is not surprising that they play crucial roles in cancer development, progression, metastasis and drug resistance. Out of around 2,600 identified human miRNAs, almost 50 % are located at fragile sites on chromosomes known for having common alterations (i.e., amplification, deletion, and rearrangements) in cancer [9]. Roles of miRNAs in cellular processes like cell cycle progression, proliferation, metabolism, apoptosis, and stress resistance [10] also cannot be overlooked as more than 60 % of human protein coding genes are predicted to be under selective pressure to be regulated by miRNAs [11]. Along with these facts, altered miRNA profiles (up/down regulated) in different cancer types suggest that these tiny molecules may have a role in cancer (therefore, the name oncomirs), and they can be classified either as tumor suppressor or oncogenic depending upon the tissue in which they are expressed and the targets they regulate [12]. Among oncogenic miRNAs, miR-17~92, miR-21, miR-155, miR-221, and miR-222 are well-studied, and their overexpression has been found in various human cancers. During lymphomagenesis, elevated miR-17~92 (a cluster of 6 miRNAs) expression has been reported which allows continuous activation of oncogenic PI3K and NF-kB signaling by suppressing negative regulators of these pathways [13]. Another oncogenic miRNA, miR-21, is upregulated in lung, prostate, breast, and pancreatic cancers compared to normal tissues [14]. miR-21 has been shown to be involved in the onset of inflammatory bowel disease (IBD)-associated colorectal carcinoma [15]. Similarly, in breast cancer, knockdown of miR-21 inhibited tumor growth and enhanced apoptosis by downregulating anti-apoptotic protein Bcl-2 [16]. Importantly, a transgenic mouse model has demonstrated that miR-21 overexpression has a causal role in tumor initiation and progression of pre-B malignant lymphoid-like tumors [17]. miR-155 is overexpressed in pancreatic cancer where it promotes tumor development by repressing the expression of tumor suppressor Tp53INP1, and oligonucleotide-mediated inhibition of miR-155 restored Tp53INP1 levels along with significant increase in apoptotic cell death [18]. Among tumor suppressor miRNAs, the most studied ones are the let-7 family miRNAs, whose expression is downregulated in different cancer types. In addition to negative regulation of Ras oncogene [19], let-7 family miRNAs target a network of cell cycle-associated genes, including E2F5, CCNA2, and CDK8, hence playing important roles in regulating multiple proliferation pathways and controlling tumor growth [20]. miR-34 is another well-studied tumor-suppressor miRNA which is directly regulated by p53 and controls p53-mediated cell death. Low miR-34 expression attenuates p53-mediated apoptosis and contributes to tumor development [21]. Other tumor suppressor miRNAs include miR-15, miR-16, miR-29, miR-124a, miR-127, miR-143, miR-145, and miR-181 [22]. Taken together, their function as negative regulators of multiple targets in biological networks and their common dysregulation in cancer make miRNAs attractive targets for cancer therapy.

This review focuses on the role of miRNAs in drug resistance, metastasis, stemness, and the interplay of these factors in tumor progression, and describes the recent developments in studying miRNA–protein interaction networks and targeting approaches in cancer. We first review recent literature with respect to the role of miRNAs in conferring resistance or sensitizing tumor cells to chemo- or targeted-therapy agents. Next, we focus on how miRNAs regulate different steps of metastasis and stemness properties of cancer cells. We then give an overview of recent studies which link miRNAs with epithelial–mesenchymal transition (EMT), stemness, and drug resistance. Finally, we focus on network-level studies to elucidate the role of miRNAs in all these cancer-relevant processes and discuss the recent developments with respect to targeting miRNAs efficiently and advance in delivery of these molecules to target tumor cells.

The role of miRNAs in drug resistance

Chemotherapy is the treatment of cancer with single or multiple cytotoxic drugs which mostly work by inhibiting the proliferation of actively dividing cells. These drugs include alkylating agents, platinum agents, nitrogen mustards, antimetabolites, anthracyclins, alkaloids, taxanes, etc. [23]. Non-specific cell targeting and late stage side effects of chemotherapy has led the way towards designing targeted therapy agents which specifically target the cancer cells by blocking the function of dysregulated proteins in oncogenic pathways. Small molecule inhibitors (mostly tyrosine kinase inhibitors, TKIs) and monoclonal antibodies are the two major classes of targeted therapy agents [24]. While monoclonal antibodies target cell-surface proteins, small molecule inhibitors can inhibit their targets inside the cell in a reversible or irreversible manner. Recently, antibody-conjugated chemotherapy agents combining targeted therapy with chemotherapy on one molecule (e.g., trastuzumab-emtansine or T-DM1 [25]) have also been developed as next-generation treatment agents and

approved for clinical use. Unfortunately, resistance to both chemotherapy and targeted therapy agents has been found inevitable and reoccurring in cancer treatment. Resistance can be divided into two groups: intrinsic (de novo) or acquired. Intrinsic resistance is an in-built, pre-existing phenotype, whereas the acquired resistance develops due to repeated use of the same drug. They both result in non-responsive treatment [26]. Possible mechanisms include decreased drug uptake, activation of detoxifying systems and DNA repair mechanisms, increased drug efflux, dodging drug-induced apoptosis, inducing secondary mutations, and activation of alternative survival pathways [27–29].

miRNAs have been associated with drug resistance to both chemo- and targeted-therapies (Table 1). Inhibition of miR-21 has been reported to increase gemcitabine sensitivity in cholangiocarcinoma and to inhibit the growth of topotecan-treated MCF7 cells [30]. Let-7e was found to be downregulated in cisplatin-resistant ovarian cancer cells due to the hypermethylation of its adjacent CpG islands compared with parental cells, and its re-expression has been shown to increase cisplatin sensitivity by reducing the levels of EZH2 and Cyclin D1 [31]. In medulloblastoma, miR-34a has been demonstrated to sensitize cancer cells to mitomycin C and cisplatin by directly targeting the oncogenic gene MAGE-A, and to induce apoptotic cell death by modulating tumor suppressor p53 levels in a positive feedback loop [32]. In another study, miR-137 has been shown to target constitutive androstane receptor (CAR), which is an important regulator of multi-drug resistance (MDR), and its overexpression sensitized neuroblastoma, hepatocellular carcinoma (HCC), and colon cancer cells to doxorubicin [33]. Notably, silencing of both Dicer and TRPB2, two key components of miRNA biosynthesis, in cisplatin-resistant adenocarcinoma cells led to reversal of cisplatin resistance without affecting the cell viability in the

Table 1 List of chemo- or targeted therapy agents which modulate the expression of given miRNAs associated with resistance to these drugs

Drugs	Downregulated miRNAs	Upregulated miRNAs	Cancer type	References
5-Fluorouracil		let-7g, miR-181b	Colon	[162]
Camptothecin		miR-506	Colon	[163]
	miR-203		Chronic myeloid leukemia	[37]
Cetuximab	let-7b, let-7e	miR-17*	Colon	[164]
Cisplatin	let-7e		Ovarian	[31]
		miR-302	Head and neck squamous CSCs	[87]
		miR-203	Breast	[165]
Doxorubicin	miR-451		Breast	[166]
	miR-137		Hepatocellular, colon	[33]
Erlotinib	miR-7		Head and neck	[167]
Fulvestrant		miR-221/222	Breast	[42]
Gefitinib	miR-146a		Non-small cell lung	[168]
Gemcitabine		miR-21	Cholangiocarcinoma	[30]
	miR-200	miR-21	Pancreatic CSCs	[89]
Irinotecan	miR-451		Colon CSCs	[88]
Letrozole		miR-128a	Breast	[169]
Mitomycin C	miR-34a		Medulloblastoma	[32]
Mitoxantrone		miR-328	Breast	[170]
Paclitaxel	let-7e	miR-130a, miR-30c, miR-335, miR-125b	Ovarian	[171]
Sunitinib		miR-21	Glioblastoma	[172]
	miR-141		Renal cell	[173]
Tamoxifen		miR-221/222	Breast	[40, 41]
	miR-375		Breast	[43]
Temozolomide	miR-145		Glioblastoma	[158]
Topotecan		miR-21	Breast	[30]
Trastuzumab		miR-21	Breast	[38]
		miR-210	Breast	[39]
Vinblastin	miR-27a, miR-451		Ovarian	[174]

absence of the drug, highlighting the general effect of the miRNA biogenesis pathway in chemotherapy resistance [34].

Similarly, miRNAs have also been shown to regulate resistance to targeted therapies. TKI imatinib is one of the iconic examples of targeted therapy agents which successfully inhibits the fusion gene BCR-ABL in patients with chronic myelogenous leukemia (CML) [35]. miR-138, a tumor suppressor that is downregulated in thyroid cancer and head and neck squamous cell carcinoma (HNSCC), was found to be upregulated upon treatment of CML cells with imatinib which resulted in an increase in imatinib-induced apoptosis and increased sensitivity to the drug [36]. This effect was mediated mainly by targeting the open reading frame (ORF) of ABL fragment in the BCR-ABL fusion protein. Similarly, Li et al. have shown that miR-203 targets the 3′-UTR of ABL and leads to sensitization of cells to imatinib in CML cells [37]. Trastuzumab is another iconic drug targeting overexpressed ErbB2 on the cell surface and has been approved by Food and Drug Administration (FDA) for breast cancer since 1998. However, development of resistance is a major problem and involvement of miRNAs in trastuzumab resistance is recently realized. Gong et al. observed overexpression of a well-known oncogenic miRNA, miR-21, in acquired trastuzumab resistant breast cancer cell lines, which confers trastuzumab resistance in both in vitro and in vivo settings. Furthermore, they demonstrated that upregulation of miR-21 is associated with poor response of patients receiving preoperative trastuzumab therapy [38]. Another study reveals that the level of miR-210 was higher in the plasma of breast cancer patients with residual disease when compared to the patients with pathological complete response under neoadjuvant trastuzumab-based chemotherapy [39]. Tamoxifen, an estrogen receptor (ER) antagonist, has been used for treating ER-positive breast cancer for decades. While it has an impressive clinical record, de novo or acquired resistance is very common. Two independent studies have associated miR-221/222 with resistance to tamoxifen, by establishing miR-221/222 downregulating the expression of ER alpha [40] and p27/Kip1 [41]. Furthermore, it has been shown that miR-221/222 also confers resistance to fulvestrant, a selective ER downregulator (SERD), by modulating both Wnt/β-catenin and TGF-β pathways [42]. We have recently demonstrated that miR-375 is downregulated in tamoxifen resistant MCF-7 cells compared with parental ones and re-expression of miR-375 sensitized resistant cells to tamoxifen partially by downregulating the oncogene metadherin (MTDH) [43]. Altogether, these reports clearly indicate the involvement of miRNAs in resistance to both chemotherapy and targeted therapy, and these miRNAs may be therapeutically modulated to sensitize tumor cells again to the drugs.

Multiple level regulation of metastasis by miRNAs

The majority of cancer deaths are not caused by primary tumors, but rather by the dissemination of the disease, i.e., the development of distant metastases. Metastasis is accomplished in two major steps: dissemination and colonization. Dissemination phase includes local invasion, intravasation into the systemic circulation, survival in the circulatory system and extravasation. Colonization phase includes the adaptation of these cells to a foreign microenvironment where the microscopic cells turn into macroscopic tumors [44]. The whole process is outcome of the interplay between genetic and epigenetic modifications in tumor as well as in the tumor microenvironment. miRNAs have recently been discovered as the key molecules regulating almost all the steps of metastasis by targeting key genes. They can either promote metastasis (in the case of, e.g., miR-373, miR-151, miR-520, miR-143, or miR-10b) or suppress the process (in the case of, e.g., miR-9, miR-139, miR-335, miR-125, or miR-206) [45, 46].

As an initial step of the metastatic cascade, cancer cells need to break away from the primary tumor, migrate and invade the surrounding tissue. In cancers of epithelial origin, increasing evidence has amounted suggesting a developmental program known as EMT which is indispensable for cancer cells to acquire properties favoring migration and invasion. During EMT, cells lose epithelial characteristics, such as apical-basal polarity and tight cell–cell adhesion, and adopt a mesenchymal phenotype characterized by extension formation, reorganization of actin cytoskeleton and decreased cell–cell adhesion, all of which contribute to the increased motility and invasiveness (reviewed in [47]). EMT is driven by a group of transcription factors that act as transcriptional repressors of E-Cadherin, including Snail (SNAI1), Slug, ZEB (ZEB1 and ZEB2/SIP1), and basic helix–loop–helix families (Twist1 and E47). miRNAs substantially contribute to EMT by regulating expression of several transcription factors and actin cytoskeleton modulators (Table 2). A subset of these miRNAs, the miR-200 family, is downregulated in metastases compared to primary tumors [48, 49] and plays a central role in the inhibition of metastasis by forming a double-negative feedback loop with ZEB1 and ZEB2, both of which are the transcriptional repressors of cell–cell contact protein E-Cadherin [50, 51]. Similarly, p53 activation leads to downregulation of EMT-inducing transcription factor Snail in different cancer types by recruiting miR-34a/b/c which directly targets the 3′-UTR of the Snail gene. The promoters of these miRNA genes are bound by Snail and ZEB1 to inhibit the expression of miR-34a/b/c, thus completing a double-negative feedback loop [52, 53]. In addition to targeting essential transcription factors regulating EMT, we and others have shown that the miR-200 family also target actin regulatory proteins such as FHOD1, PPM1F and moesin to inhibit EMT [54, 55].

Table 2 List of miRNAs and their targets involved in regulation of different stages of metastasis in different cancer types

miRNA	Target (Direct/*Indirect*[a])	Suppress/promote	Cancer type	Metastasis stage	References
miR-200c	BMI1	Suppress	Head and neck	EMT	[91]
miR-34	Snail	Suppress	Breast, lung, colon	EMT	[52, 53, 96]
miR-29b	ANGPTL4, LOX, MMP2, MMP9, VEGFA, PDGF	Suppress	Prostate/hepatocellular	EMT	[175, 176]
miR-30a	Snail	Suppress	Non-small cell lung	EMT	[177]
miR-200 family	TGFβ2, ZEB1, ZEB2	Suppress	Pancreatic, colorectal, breast, lung	EMT, invasion	[48, 50, 51]
miR-22	TET family members	Promote	Breast	EMT, invasion	[95]
miR-200b	Moesin	Suppress	Breast	EMT, invasion	[55]
miR-200c	FHOD1, PPM1F	Suppress	Breast	EMT, invasion, migration	[54]
miR-7	KLF4	Suppress	Breast	Invasion	[93]
miR-135b	LATS2, β-TrCP, NDR2, LZTS1	Promote	Non-small cell lung	Invasion, migration	[178]
miR-10b	HOXD10	Promote	Breast	Invasion, migration	[179]
miR-34a	CD44	Suppress	Prostate CSCs	Invasion, migration	[94]
miR-21	Pdcd4	Promote	Lung	Invasion, intravasation	[56]
miR-520/373 family	TGFBR2 (NF-κB and TGF-β pathways)	Suppress	Breast	Invasion, intravasation	[60]
miR-493	IGF1R	Suppress	Colon	Extravasation	[58]
miR-148b	ITGA5, ROCK1, PIK3CA/p110α, NRAS, CSF1	Suppress	Breast	Invasion, extravasation, survival to anoikis	[59]
miR-214	TFAP2	Promote	Melanoma	Extravasation	[180]
miR-31	*Integrin α5, Radixin, RhoA*	Suppress	Breast	Extravasation, colonization	[57]
miR-200 family	Sec23a	Promote	Breast	Colonization	[62]
miR-612	Akt2	Suppress	Hepatocellular	Colonization	[46]

[a] Denotes that indirect targets in the given study are shown in *italics*

Besides regulating EMT to drive cancer cells more motile and invasive, miRNAs also regulate cell intravasation to the circulatory system as well as the post-intravasation steps including extravasation and initial survival at the distant tissue. Asangani et al. have shown that inhibition of miR-21 in colorectal cancer cells reduced intravasation and lung metastasis in a chicken embryo metastasis assay by upregulating the expression of its target Pdcd4, a tumor suppressor gene [56]. miR-31 has been demonstrated to regulate several post-intravasation steps including intraluminal viability, extravasation, and survival at distal tissue in addition to the invasion and metastatic colonization steps by simultaneous targeting of three key genes: integrin α5 (ITGA5), radixin (RDX), and RhoA (RHOA) [57]. Recently, Okamoto et al. identified miR-493 as an inhibitor of the settlement of colon cancer cells to the liver parenchyma in a functional miRNA screen. They demonstrated that miR-493 directly targets the receptor tyrosine kinase IGF-1R and this in turn leads to the apoptosis of metastasized cells [58]. Very recently, it is reported that miR-148b regulates both extravasation and survival of breast cancer cells in circulation in both in vitro (apoptosis assay) and in vivo (tail-vein injection for extravasation to the lungs) experiments. Data indicated that miR-148b reduced both extravasation and survival by regulating metastatic dissemination in several steps [59]. Recently, we have shown that miR-520/373 family inhibits both in vitro cell invasion and in vivo intravasation of highly invasive ER (−) breast cancer cells. Decreased expression of miR-520c was found to be correlated with the lymph node metastasis of ER (−) breast cancer patients [60].

The final step of metastasis is successful colonization of tumor cells at a distal organ site. Different tumors have different preferences for the site of metastasis. This observation forms the basis of century-long seed-and-soil hypothesis where “seed” represents the “cancer cells” and the “soil” stands for the “tumor microenvironment” [44]. Consistent with this hypothesis, breast cancer cells metastasize frequently in bone, lungs, liver, and brain, while pancreatic cancers preferentially metastasizes to liver and lungs (reviewed in [61]). Few studies have focused on the roles of miRNAs in the colonization step. Korpal et al. demonstrated that miR-200 promotes the colonization of breast cancer cells by directly targeting the Sec23a gene which is involved in the secretion of metastasis-suppressive proteins. This study is also a good example of dichotomous function of miRNAs in the initiation (i.e., inhibiting EMT) and final colonization (i.e., promoting colonization) steps of metastasis [62]. Recently, miR-612 is suggested to suppress the colonization of HCC cells to the

lungs [46]. Finally, as discussed above, miR-31 regulates colonization by targeting ITGA5, RDX and RHOA protein network.

Several recent studies demonstrated that miRNAs can sensitize cells to given drugs by modulating the EMT step of metastasis, implying an intriguing link between metastasis and drug resistance (Fig. 1, Supplementary Table 1). miR-200c, a downregulated miRNA in melanocytes, targets BMI-1 when overexpressed, leading to increased E-cadherin levels and thereby inhibiting metastatic potential of cancer cells. In the same study, it was also shown that overexpression of miR-200 inhibits cell proliferation and resistance to cisplatin, PLX4720 and U0126 [63]. Re-expression of miR-200c in non-small cell lung cancer (NSCLC) inhibited invasion by maintaining epithelial phenotype and improved the sensitivity of cancer cells to cetuximab and cisplatin [64]. Treatment of gemcitabine resistant pancreatic cancer cells with natural agents (DIM and isoflavone) induces miRNAs of both miR-200 and let-7 families, which restore the epithelial state and improve the sensitivity to gemcitabine [65]. As previously discussed, we have recently demonstrated that re-expression of miR-375 in tamoxifen resistant MCF-7 cells sensitized resistant cells to tamoxifen by reversing the mesenchymal phenotype of resistant cells to more epithelial one [43]. miR-23, an oncogenic miRNA, has been shown to be upregulated in lung adenocarcinoma cells driving EMT. Downregulation of this miRNA in A549 lung cancer cells restored epithelial phenotype along with enhancing sensitivity for gefitinib [66]. miR-216a/217 target PTEN and SMAD7, antagonists of PI3K and TGF-β signaling pathways, respectively, and assist in maintaining drug resistance and high metastatic potential in HCC. Inhibiting miR-216/217 or overexpressing their targets rescued EMT and sensitized cancer cells to sorafenib [67]. Altogether, these findings suggest that miRNAs regulate metastasis at multiple steps by modulating different components of the cellular networks and modulation of EMT by miRNAs can sensitize the cells to chemo- or targeted-therapy agents.

miRNA regulation of stemness, EMT, and drug resistance in cancer

Stemness is the ability of a cell to differentiate into any other cell type (potency) while maintaining an undifferentiated state (self-renewal) [68]. Adult stem cells have been found in almost every tissue type and organ maintaining tissue homeostasis and in regenerating tissues after injury or damage [69, 70]. The connection between stemness and tumor initiation was first shown when a population of leukemic cells expressing surface markers identical to hematopoietic stem cell (HSC) markers (CD34+/CD38−) was found to initiate acute myeloid leukaemia tumors in NOD/SCID mice [71]. Later on,

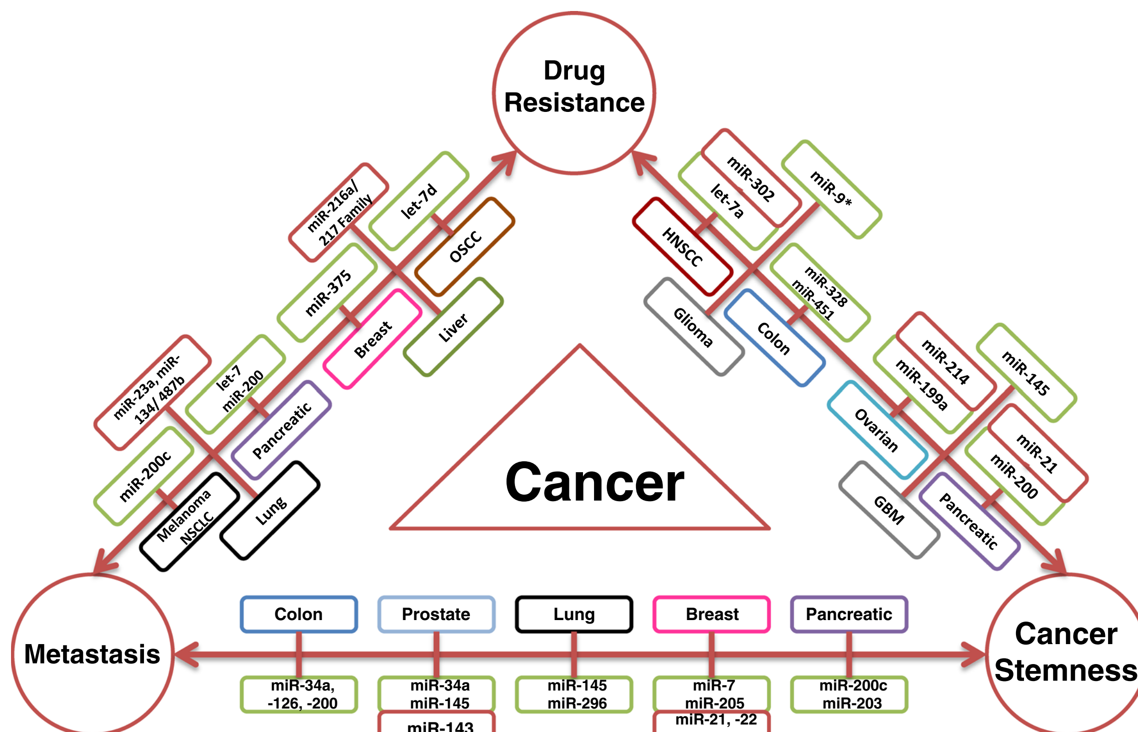

Fig. 1 MicroRNAs play master role in the interplay of drug resistance, metastasis and cancer stemness. A brief outlook of miRNAs reported to co-regulate any two of these mechanisms in a specific cancer type are shown [43, 63–67, 79, 86–89, 92–96, 150–161] where oncogenic (upregulated) miRNAs are marked with red and tumor suppressor (downregulated) miRNAs are marked with green background. *OSCC* oral squamous cell carcinoma, *NSCLC* non-small cell lung carcinoma, *HNSCC* head and neck squamous cell carcinoma, *GBM* glioblastoma

cancer stem cells (CSCs), also known as tumor initiating cells (TICs), were identified in a number of solid tumors including breast, brain, colon, prostate and pancreatic cancer [72–76]. Although involving similar pathways (e.g. Wnt, Notch and TGF-β signaling), CSCs distinguish themselves from normal stem cells by altered molecular profiles. For example, leukemic stem cells require loss of tumor suppressor PTEN to be distinguished from HSCs [77]. CSCs represent small population in tumor [78], but in addition to maintaining stemness, they also take part in driving tumor growth, metastasis, and drug resistance [79]. Several miRNAs have been shown to regulate stemness of different cancer types. For instance, three miRNA clusters (miR-200c-141, miR-200b-200a-429, and miR-183-96-182) have been found downregulated in both normal and CSCs to overexpress Bmi1 which plays a role in stem cell renewal [80]. Recently, a comparative expression analysis of CD44+ (a marker for CSCs) and CD44− colorectal cancer cell lines revealed association between miR-203 downregulation and cancer stemness. In CD44+ cells, hyluranon (HA) signaling (HA/CD44 interaction) triggers c-Src kinase mediated nuclear accumulation of Snail, an inhibitor of miR-203, resulting in enhanced stemness [81]. Increased expression of miR-21 and miR-302 and decreased levels of let-7a, miR-372, miR-373, and miR-520c-5p were observed in CSCs when compared with cancer cells in a human gastric cancer cell line, suggesting differential expression of miRNAs in CSC and other cancer cell populations [82]. Ji et al. identified higher expression of miR-181 in EpCAM+/AFP+ stem cell population of HCC. Its inhibition resulted in a decrease in this population and tumor initiating capacity of the cells [83]. The oncogenic miR-21 enhanced stem-like properties in colorectal cancer by downregulating transforming growth factor beta receptor-2 (TGFβR-2) along with activating the oncogenic Wnt signaling pathway [84].

CSCs are thought to be responsible for the observed resistance to therapy and metastasis. Several miRNAs have been reported to regulate stem cell properties and drug resistance concomitantly (Fig. 1, Supplementary Table 2). CD133 is a well-established CSC marker in brain tumors including glioblastoma (GBM) [85]. Ectopic expression of miR-145 in GBM-CD133+ cells resulted in their differentiation into CD133- non-CSCs and thereby reduced resistance to temozolamide [86]. Due to the Hyaluronan (HA) activation of CD44v3 (an HA receptor) leading to nuclear accumulation of oncogenic transcription factors (Nanog, Oct4, Sox2), CSCs in HNSCC display upregulated miR-302 expression which, in turn, upregulates several survival proteins responsible for clonal formation, self-renewal and cisplatin resistance [87]. miR-451 has been shown to be downregulated in drug resistant colon CSCs, and its restoration antagonized irinotecan resistance and the self-renewal capacity via indirect targeting of COX-2-mediated Wnt-signaling [88]. Decreased miR-200 and increased miR-21 levels have been observed in gemcitabine-resistant pancreatic CSCs, and the treatment of these CSCs with synthetic compound-CDF resulted in tumor inhibition through reversing miR-200 and miR-21 expression, which modulates stemness-related pathways [89]. miR-26a has been demonstrated to be lost in pancreatic CSCs and its re-expression decreased expression of CSC markers (EpCAM and EZH2) in pancreatic cancer. In this respect, it was an interesting observation that pancreatic CSCs treated with metformin showed increased expression of six miRNAs including miR-26a and decreased expression of different CSC markers [90].

Similar to the critical role of miRNAs in the interplay of stemness and drug resistance, miRNAs also co-regulate stemness and metastatic capabilities of the cells, especially by regulating the EMT process (Fig. 1, Supplementary Table 3). It has been shown that the expression of miR-200c was downregulated, while its target Bmi1 was upregulated in ALDH1+/CD44+ HNSCC cells compared with other cell populations. Notably, overexpression of miR-200c in these CSC populations inhibited CSC-like properties, decreased the expression of EMT-associated ZEB1 and Snail, and inhibited the lung metastasis capability of the cells [91]. Importantly, ZEB1 has been shown to work in a feedback loop with miR-200 and also to play a role in repression of stemness inhibiting miRNAs (e.g. miR-203). This way ZEB1 controls both EMT-activation and cancer stemness maintenance [92]. In another study, CSC population (CD24−/CD44+/ESA+) isolated from highly metastatic breast cancer cells were found more capable of metastasis than the non-CSC population. miR-7 was shown to be downregulated in bone and brain metastases derived from these CSCs and its re-expression in breast CSCs suppressed brain metastasis by downregulating KLF4, a stemness related gene [93]. miR-34a has been shown to be downregulated in CD44+ prostate CSCs and its re-expression or knocking down of its direct target CD44 suppressed both tumor growth and metastatic capacity in prostate cancer [94]. Very recently, Song et al. demonstrated that miR-22 promotes EMT and increases stem cell population of mammary tumors, resulting in increased metastasis potential of different transgenic mammary tumor models. Mechanistically, miR-22 exerts its metastatic properties by directly targeting the TET family of methylcytosine dioxygenases, leading to reduced expression of anti-metastatic miRNAs, particularly the miR-200 family [95]. Finally, another recent study demonstrated that disruption of Dicer in colorectal cancer cells resulted in decreased expression of key tumor suppressor miRNAs, miR-200 family and miR-34a, which, in turn, led to the enrichment of stemness properties and the induction of EMT with increased liver metastasis of colorectal cells [96]. In conclusion, miRNAs play crucial roles in maintaining cancer stemness, associated-drug resistance and metastasis, and targeting miRNAs regulating the interplay of stemness with metastasis

and drug resistance might be more effective in preventing metastasis or overcoming resistance to both chemo- and targeted-therapy.

Systems biology approaches to identify miRNA–protein interaction networks regulating drug resistance and metastasis

Our understanding of miRNAs and their functions in drug resistance, metastasis, and cancer stemness is continuously amounting but still limited. The observation that each miRNA can have many targets and that each gene can be regulated by multiple miRNAs implies that studies designed with "one miRNA vs. one target" concept are likely to be flawed. Therefore, cancer researchers have long embraced the concept of systems biology and combined wet-lab experiments with bioinformatics analysis in order to elucidate how miRNAs operate on the network level by regulating many targets at a time.

The necessity and usefulness of studying miRNA regulation of genes or proteins at network level have been demonstrated by several groups. Pencheva and Tavazoie reported in a recent review that a number of different miRNAs promote or suppress metastasis by forming functional networks with their targets in three fashions: cell-autonomous, cell-non-autonomous, or a mixed of the two [97]. The more than one-hundred studies on miRNA–protein networks that were reviewed do not only offer novel insights into their roles in metastasis, but also stimulate and accelerate the discovery of genes and pathways that control various aspects of the biological process. In another study focusing on general principles of systems biology approaches to study miRNAs in cancer, Vera et al. promoted the idea that systems biology and molecular biology approaches must be integrated. To support this idea, they demonstrated the feasibility of using a mathematical tool (ordinary differential equations, ODE) to predict functions of miRNA by modeling and simulation [98]. Another study by Aguda et al. demonstrated the feasibility of using mathematical models to elucidate a miRNA regulated cancer network where a miRNA cluster, miR-17-92, inhibited the expression of MYC and E2F transcription factors which, in turn, induced the expression of miR-17-92 cluster providing a negative feedback loop. In this study, the generated model predicted that miR-17-92 plays an important role in regulating the expression levels of these proteins [99]. Furthermore, a recent study generated a kinetic model using the knowledge on E2F, p73 and miR-205 and proposed that high E2F1, low miR-205 and high ErbB3 levels make the cancer cells resistant to both genotoxic and cytotoxic drugs [100].

To study microRNAs' roles in the context of biological networks experimentally, either forward-engineering or reverse-engineering approaches have been used (Fig. 2). Forward-engineering approaches are forward-genetics experiments where one or more miRNAs (or genes) are either inhibited or over-expressed by genomic integration [101], chemical inhibitors or mimics [102], expression constructs [103] or other tools. To identify target genes and study the regulatory effects (amplitude, dynamics, etc.), expression profiles of genes or miRNAs are measured. Omics technologies, such as gene/miRNA microarrays [104], next-generation sequencing [105], protein arrays [106], and mass spectrometry [107], are often used to identify potential targets, since they can cover a large number of genes in an almost unbiased way. The obvious challenge is that except for special cases, it is difficult to dissect direct from indirect effects in such experiments. Therefore, computationally predicted microRNA/gene target pairs are often used to filter large number of potential hits (reviewed in [108, 109]). Furthermore, downstream experiments are usually required, often in a low-throughput manner, to validate the findings. For instance, to validate direct miRNA–gene regulations, one possibility is to couple point mutation of miRNA-binding regions in 3′-UTR of target genes with luciferase assays [43] [54]. It is expected that technological development will strongly boost the forward inference approaches. Future experiments may bring more power and insight by becoming more multiplex [110] and by integrating multilayer and heterogeneous data [111]. Increasing throughput and lower costs will allow more data generated to fine-map expression of miRNAs and their targets in different tumor types and disease stages [112, 113]. New technologies such as next generation sequencing [114] and high-throughput mass spectrometry [115] and other quantification methods [106] are pushing the fronts of forward-engineering approaches.

In contrast to forward-engineering approaches, reverse-engineering approaches often deal with expression profiles that were not generated by specific perturbation of genes or miRNAs. A typical example is paired expression profiling of mRNA and miRNA in drug-sensitive and drug-resistant cell lines. In such settings, the primary goal is to recover miRNA-gene, gene-gene and though less commonly, miRNA-miRNA regulatory relationships. Various network-inference algorithms such as correlation-based methods [116], Bayesian network methods [117], regression-based methods [118], and modeling-based approaches [119] have been applied to infer the structure of miRNA–protein networks.

Finally, combining forward and reverse engineering approaches allows biologists to construct comprehensive regulatory networks incorporating transcription factors (TFs), target genes, miRNAs, and chemical compounds. Many algorithms and tools that are available to study both global and local properties of networks (reviewed in [120, 121]) have been applied to miRNA-protein interaction networks. For instance, it has been observed that miRNAs and TFs are often involved in network motifs, namely local network structures that are more abundant than a random network model would have predicted, such as feedback loops and feed-forward loops [122, 123]. Applications exploiting these properties are emerging to improve therapeutic regimens

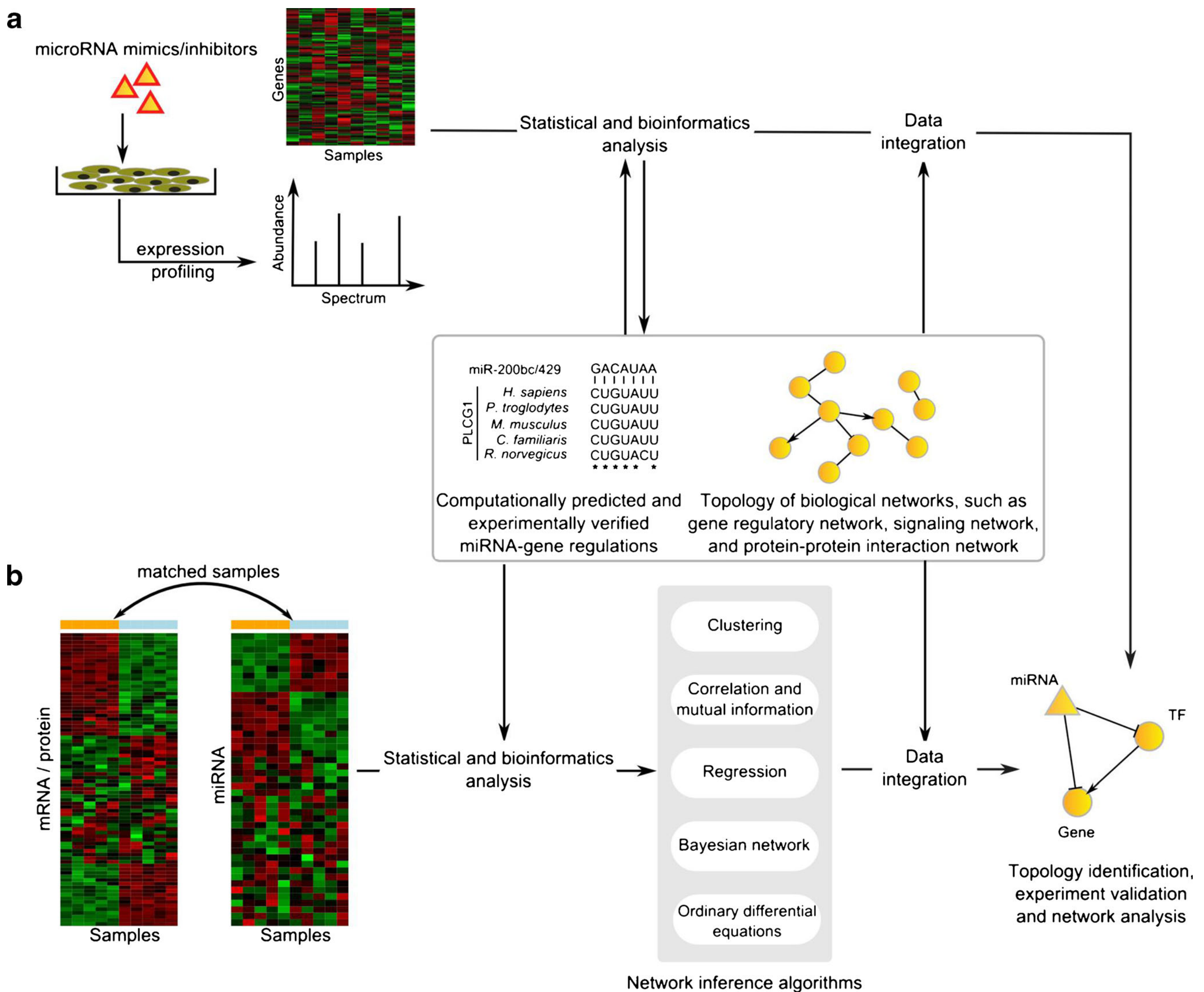

Fig. 2 Workflow diagrams of forward-engineering (**a**) and reverse-engineering (**b**) approaches to identify the "wiring-diagram" of microRNA-protein networks. Forward-engineering methods generally begin with targeted perturbation of microRNA (illustrated here as example) or gene expression. Omics technologies such as mRNA and protein expression profiling are coupled with in silico methods to detect targets or regulators of microRNAs. In contrast, reverse-engineering methods attempt to make inference about the network structure by applying machine learning algorithms to paired expression data of microRNA and mRNAs (or proteins). In either case, prior knowledge can be integrated to the analysis pipeline, and often downstream experiments are required to validate the findings. The aim of both types of approaches is to build a comprehensive microRNA-protein network. Its local and global properties provide insights to the network-level mechanism of microRNA regulation, and create opportunity for novel therapeutic interventions against cancer

and to enable new therapeutics against cancer. For instance, Zhang et al. reported the blockage of a miR-21/EGFR regulatory feedback loop by combining nimotuzumab (an EGFR targeting antibody) and a miR-21 inhibitor augments anti-EGFR therapy in GBM [124]. In another study, disruption of the MYC-miR-26a-EZH2 feed-forward loop with small-molecule compounds was found to suppress lymphoma growth and clonogenicity in aggressive lymphoma cells [125]. Both integrative basic research and translational studies are further called to elucidate miRNA regulatory networks and to transform knowledge in network biology of miRNAs into biomarkers and therapies. Researchers can benefit from evolving technologies, amounting data shared in public databases such as Pharmaco-miR [126], miR2Disease [127], and OncomiRDB (http://bioinfo.au.tsinghua.edu.cn/member/jgu/oncomirdb), novel bioinformatics algorithms and new experimental systems such as high-throughput in vivo screening [128].

Promises and challenges in targeting miRNAs in cancer

Dysregulated miRNA expression and their roles as oncogenes or tumor suppressors by targeting protein networks make miRNAs a promising option to sensitize cancer cells to

therapy or to prevent metastasis. The notion of modulating miRNAs in cancer comes from the analogy of killing two birds (drug resistance and metastasis) with one stone (miRNA). As we have discussed above, inhibition of oncogenic miRNAs or replacement of downregulated miRNAs by exogenous expression in different experimental models suppressed tumor growth and metastasis, and even sensitized refractory tumors to different chemotherapy or targeted therapy agents.

There are several strategies being evaluated to target miRNAs or use them as targeting agents in cancer, such as (a) inhibition of oncogenic miRNAs by antisense DNA oligonucleotides, antagomirs, locked nucleic acids (LNAs), RNA sponges, or miR-masking; (b) exogenous expression of tumor suppressor miRNAs; and (c) targeting miRNAs by using small molecules (reviewed in [129]). Early experiments in *C. elegans* demonstrated the ineffectiveness of using unmodified antisense DNA oligonucleotides. However, 2′-O-methyl or 2′-O-methoxyethyl conjugated oligonucleotides significantly improved binding specificity and affinity for RNA [130]. Later on, “antagomirs” with 2′-O-methyl modifications were developed against miR-122, an abundant miRNA in liver. In vivo injection efficiently downregulated miR-122 expression in mouse liver after 24 h [131]. This method is specific, efficient, and long-lasting, and has led to more antogomirs that are being tested. Another approach to inhibit miRNAs uses LNAs which are oligonucleotides with ribose ring “locked” by methylene bridge between 2′-O and 4′-C atoms. It improves target specificity and affinity towards complementary miRNA sequences. Recently, LNA-mediated downregulation of miR-155 has been shown to decrease tumor growth in chronic lymphocytic leukemia (CLL) [132]. Complementarily, expression of tumor suppressor miRNAs can be restored by direct or vector-based transfection of miRNA mimics. For example, intranasal administration of let-7 has been shown to reduce the tumor formation in lungs of animals expressing G12D-mutated K-Ras oncogene [133]. Similarly, re-expression of miR-29, a miRNA which sensitizes HCC cells to doxorubicin, attenuated the ability of HCC cells to form tumors in mice [134]. Furthermore, systemic (intravenous) delivery of miR-141 or miR-219 inhibits osteolytic bone metastasis in vivo [135]. Last but not least, small molecule inhibitors are being searched for to target miRNAs. A luciferase-based screening with a library of more than 1,000 compounds, for instance identified azobenzene as a potent inhibitor of oncogenic miR-21 expression in HeLa cells [136].

In addition to the development of diverse strategies to target or replace miRNAs, there have been a number of studies aiming to improve the delivery of miRNAs in an efficient and specific manner to target tumor tissues. Initially, vector-based delivery approaches including both adenoviral- and lentiviral-based delivery approaches were proposed to replace the tumor suppressor miRNAs such as, miR-26a [137] and let-7 g [138]. However, they are associated with increased toxicity and induction of immune response, which limit the translation into clinics [139]. As an alternative approach, non-viral delivery approaches e.g. lipid-based nanoparticles containing miRNAs have been developed. Piao et al. demonstrated that lipid-based delivery of miR-107 precursors led to efficient reduction of multiple targets’ expression and tumor growth in HNSCC [140]. Furthermore, several other nanoparticle-based miRNA delivery approaches have been reported. For example, Babar et al. reported that PLGA nanoparticles conjugated to a cell-penetrating peptide efficiently delivered the anti-miR-155 into tumor cells and inhibited the growth of pre-B-cell tumors in vivo [141]. In addition to nanoparticles for miRNA delivery, very recently, Ohno et al. demonstrated that EGF-like peptide containing exosomes delivered let-7a to EGFR-overexpressing breast tumors in vivo [142]. Compared with vector-based delivery methods, these synthetic delivery systems are more favorable due to simplicity, allowing more control over the size of particles and the distribution of molecules, while eliciting less immunogenicity, However, currently, these synthetic systems have relatively low efficiency (reviewed in [139]), and it lacks long-time studies with regard to their toxicity. Overall, specific inhibition or replacement of miRNAs and efficient delivery to target sites may hold the promise to sensitize cancer cells to therapy and to prevent metastasis; however, much work has yet to be done to prove their efficacy, safety and clinical applicability.

Conclusion and perspectives

We have reviewed here the role of miRNAs in drug resistance and metastasis, both of which are the major causes of cancer-related deaths, and their interplay with cancer stemness. As miRNAs can have a huge impact on the gene expression at the global scale and they are dysregulated in cancer, targeting or replacing miRNAs have great promise for sensitizing cancer cells to therapy agents and for preventing the metastasis. However, before translation of pre-clinical findings into clinics, there need to be significant improvements in several aspects of studying the miRNAs. First, clinically-relevant models and scenarios could be prioritized when studying the role of miRNAs in drug resistance. Most of the studies examining the sensitizer role of miRNAs have been using two dimensional cell culture systems. There established cancer cell lines are continuously cultured in the presence of drug and resistance develops after several months. Such models miss the contribution of tumor microenvironment and oversimplify the therapy regimens used in the clinics. For this purpose, transgenic mouse models or syngeneic tumor transplantation models with intact tumor microenvironment [143] can be used to develop drug resistant animal models. In these

models miRNAs can be modulated stably and/or inducibly, and the sensitizer effects of miRNAs can be studied. Alternatively, patient-derived xenografts with known therapy history [144] can be used to deliver miRNAs into tissues and to study whether miRNAs can sensitize refractory tumors to the therapy. Secondly, although the role of miRNAs has been relatively well-studied in the initial phase of metastasis, particularly the EMT process [145], how different tumor microenvironment orchestrates the organ-specific metastasis by secreting different miRNAs and whether these miRNAs are really effective in blocking the colonization of cancer cells are still under-studied. Another area where studying the role of miRNAs is limited is cancer stemness which has been hampered by the low number of stem-like cells in heterogeneous tumor population [146]. This may be circumvented by newer technologies such as high-content screening and single-cell sequencing.

Systems biology approaches to integrate miRNAs into gene or protein networks have been developed and being utilized in recent studies [110, 125, 147]. In order to predict the outcome of miRNA modulation in a cell system, models integrating miRNAs into gene or protein networks have to be developed and loss-of-function or gain-of-function simulations should be performed similar to the ones done for protein-coding genes. These models will help understand the mechanism of miRNA regulation at the global scale, and eventually predict efficacy and potential side effects of modulation of miRNAs. Furthermore, more research on the tumor tissue-specific delivery methods for targeting tumors with miRNAs using specific tumor markers on the carriers [142] are needed to reduce the side-effects of treatments. Although we did not focus on the biomarker aspect in this review, miRNAs have a good potential to predict therapy response and outcome due to their stability and ubiquitous presence in body fluids [148, 149]. There are currently more than dozens of clinical trials using miRNAs or proteins in miRNA biogenesis as biomarkers of tumor progression, metastasis and response to chemotherapy or targeted therapy agents (www.clinicaltrials.gov). More research with larger cohorts and comprehensive designs should be carried out to establish miRNAs as clinically useable biomarkers.

In conclusion, considering the complexities intrinsic to cancer genome, miRNA regulation, and many aspects of fight against cancer—especially the ones we discuss in this review, drug resistance, metastasis, and stemness—we believe that the research community urgently needs better understanding of miRNA–protein interaction networks. We therefore propose that multi-disciplinary systems approaches integrating genomics, genetics, proteomics, and bioinformatics may hold the key.

Acknowledgments We thank Gülce Itır Perçin, Merve Bal, Mustafa Can Kiren and Ezgi Bağırsakçıoğlu for helping us prepare the figures and tables. We thank Dr. Martin Ebeling for insights and discussions on network-based analysis. We apologize to the many authors whose work could not be cited directly because of space limitations. This work was supported by EMBO Installation Grant (ÖŞ), Bilkent University start-up funds (ÖŞ) and the scholarship from Higher Education Commission of Pakistan (UR).

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