Chapter 10
MicroRNA Based Therapeutic Strategies for Cancer: Emphasis on Advances in Renal Cell Carcinoma

Shahana Majid and Rajvir Dahiya

1 Introduction

In recent years, it has become increasingly apparent that the non-protein-coding portion of the genome is of crucial functional importance for normal development, physiology and disease [1]. The functional relevance of the non-protein-coding genome is particularly evident for a class of small non-coding RNAs (ncRNAs) called microRNAs (miRNAs) [2, 3]. miRNAs are small ncRNAs of ~22 nucleotides that mediate post-transcriptional gene silencing by controlling the translation of mRNA into proteins [2, 3]. miRNAs are estimated to regulate the translation of more than 60 % of protein-coding genes. They are involved in regulating many processes, including proliferation, differentiation, apoptosis and development. The frequent aberrant expression and functional implication of miRNAs in many human diseases have highlighted their potential as preferred drug targets [4, 5]. Notably in cancer, certain miRNAs meet the stringent criteria for being ideal therapeutic targets since they by function as bona fide oncogenes and tumor suppressors. 'Oncogene addiction', a term previously reserved for protein-encoding oncogenes, has been extended to miRNAs [6]. Since mammalian microRNAs do not require perfect complementarity for target recognition, a single microRNA is able to regulate multiple, perhaps hundreds of, messenger RNAs [7, 8]. MicroRNAs may impact a given phenotype through regulation of a single key target or through concomitant regulation of a subset of targets. In some instances a phenotypic change can be explained by partial suppression of a single target, as illustrated by the ability of miR-150 to control lymphocyte development by regulating the expression of the seed matched target c-Myb [9]. For other microRNAs the story is more complex, with the phenotype being controlled by the coordinated suppression of multiple targets [10]. MicroRNAs modestly down-regulate individual

S. Majid (✉) • R. Dahiya
Department of Urology, Urology Research Center (112F), VA Medical Center and UCSF, 4150 Clement Street, San Francisco, CA 94121, USA
e-mail: smajid@urology.ucsf.edu

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mRNAs (30–50 % down-regulation), yet this degree of target silencing, is sufficient to induce phenotypic changes. Since cancer is a heterogenic disease that cannot be successfully treated by targeting a single gene of interest [11–13], the ability of miRNAs to regulate multiple pathways deregulated in cancer may be key to therapeutic success. Here, we focus on targeting these miRNAs using novel therapeutic strategies for cancer with emphasis on advances in renal cell carcinoma.

2 Disruption of miRNAs in Cancer

In human cancer, miRNA expression profiles differ between normal tissues and the tumors that are derived from them and also between tumor types. miRNAs can act as oncogenes or tumor suppressor genes and can play key roles in tumorigenesis [5, 14]. Transcriptional regulation of miRNAs by oncogenes and tumor suppressor genes provided initial evidence that miRNAs are linked to cancer. In parallel, strong genetic evidence has emerged showing that miRNA genes could be amplified, deleted, or epigenetically regulated in cancer in the same way that canonical cancer genes are affected by somatic or germline mutations. Furthermore, dysregulation of miRNAs in cancer can occur through epigenetic changes such as promoter CpG island hypermethylation. For example, in the case of the miR-200 family [15], genetic alterations can affect the production of the primary miRNA transcript, their processing to mature miRNAs and/or interactions with mRNA targets [16]. From a genetic standpoint, one of the first associations to be observed between miRNAs and cancer development was miR-15 and miR-16 dysregulation in most B cell chronic lymphocytic leukemias as a result of chromosome 13q14 deletion40. Finally, over-expression/down-regulation of certain miRNAs in advanced versus early-stage cancers lead to the idea that some miRNAs might confer metastatic properties and function as metastamiRs in cancer [17].

3 Therapies Targeting miRNAs

Depending on miRNA function and its status in diseased tissues, there are two approaches to developing miRNA-based therapies: antagonists and mimics. MicroRNA antagonists are generated to inhibit miRNAs that acquire a gain of function in human disease. In contrast, miRNA mimics are used to restore miRNAs that show a loss of function. This approach, also known as miRNA replacement therapy, has attracted much interest as it provides a new opportunity to therapeutically exploit dysregulated miRNAs [18] (Fig. 10.1).

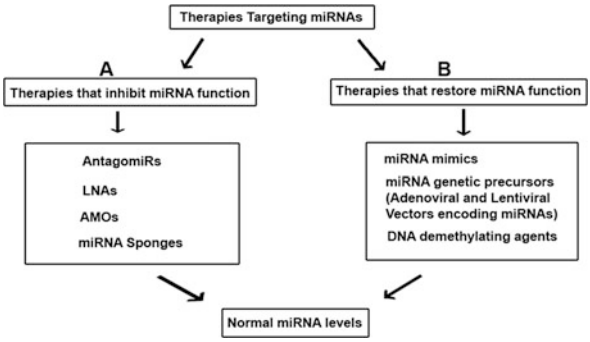

Fig. 10.1 Therapies targeting miRNAs in cancer. (**a**) Strategies that inhibit oncogenic miRNAs include synthetic antisense oligonucleotides (ASOs) or modified ASOs that incorporate chemical groups to improve the stability and efficacy, such as anti-miRNA oligonucleotides (AMOs), antagomirs or locked nucleic acids (LNAs), miRNA sponges that contain multiple artificial miRNA binding sites that act as sponges for the cognate miRNA, preventing its association with endogenous targets. (**b**) Strategies that restore miRNAs include miRNA mimics, virus delivery systems: for example, adenovirus and lentivirus-associated vectors that code for downregulated miRNAs and epigenetic drug treatments for reactivating the transcription of silenced miRNA using DNA demethylating agents

3.1 Therapies that Inhibit miRNA Function

The knowledge that miRNAs regulate their targets through base pairing has led to the use of antisense oligonucleotides (ASOs) to inhibit miRNA function therapeutically. ASOs inhibit miRNA targets based on base pair complementarity. Three main classes of ASOs that have been developed are locked nucleic acids (LNAs), anti-miRNA oligonucleotides (AMOs) and antagomirs that incorporate different chemical modifications to increase stability and efficacy [19–21]. MiRNAs with oncogenic capacity can be deactivated or silenced by various strategies given below.

3.1.1 Anti-miRNA Oligonucleotides (AMOs)

AMOs are single-stranded molecules that form direct complementarity and thus inhibit a specific miRNA [22].

3.1.2 Antagomirs

Antagomirs are 21–23 long ribonucleotide chains wherein 2′-hydroxyl on the ribose is replaced with a 2′-O-methyl group. The backbone is also modified by replacing some of the phosphodiester linkages by phosphothioate ones. These two modifications greatly improve biostability yielding oligonucleotides more resistant to

degradation. To enhance cell penetration, the single-stranded modified RNA is conjugated to a molecule of cholesterol. In addition, antagomir sequences are not perfectly complementary to their targeted miRNA and have base modifications and mispairings that inhibit cleavage by Ago proteins. This results in a near-irreversible binding between the miRNA and the antagomir, which prevents miRNA binding to its cognate mRNA target. The first *in vivo* delivery of antagomirs was via intravenous injection of antagomirs against miR-16, miR-122, miR-192 and miR-194 that resulted in long-lasting reduction of these miRNAs in several organs [19].

3.1.3 Locked Nucleic Acids (LNAs)

LNAs have a methylene bridge that functionally locks ribose conformation. This change results in increased binding affinity and stability [22]. LNAs can be mixed with regular RNA and DNA nucleotides yielding more stable oligomers [23].

3.1.4 Multiple-Target Anti-miRNA Antisense Oligodeoxyribonucleotide (MTg-AMO)

Silencing of a single miRNA might not be sufficient in all cases owing to the pleiotropic and multifaceted biology of cancer cells. Recent research in this field suggests that several miRNAs can be simultaneously inhibited using single ASOs that are targeted against multiple miRNAs. In this approach, multiple antisense units are engineered into a single unit called a multiple-target anti-miRNA antisense oligodeoxyribonucleotide (MTg-AMO) [24]. One MTg-AMO was designed to target three oncogenic miRNAs, miR-21, miR-155 and miR-17-5p, that are overexpressed in many tumors. Use of this MTg-AMO resulted in increased inhibition of cancer growth [24].

3.1.5 miRNA Sponges

Another innovative strategy involves expressing competitive inhibitors of miRNA function. These ‘miRNA sponges’ are vectors containing multiple artificial miRNA binding sites that are placed under the control of strong promoters to produce large quantities of transcript. They act as sponges for cognate miRNAs, preventing their association with natural targets [25, 26]. This strategy has been used, for example, to inhibit miR-9 in highly malignant cells, demonstrating the role of miR-9 in metastasis [26].

3.1.6 Nanoparticles

Nanoparticle formulations have been used primarily *for in vitro* delivery of siRNAs. Few studies to date have used this technology for miRNA delivery. Systemic delivery of miR-34a in a lipid-based vehicle by intravenous injection was used to block proliferation of grafted subcutaneous human lung cancer cells, inducing tumor cell death without triggering toxicity in liver, kidney or heart, as shown in a study from Mirna Therapeutics, Inc. Furthermore, the formulated miR-34a didn't trigger an immune response [27]. Chen et al. demonstrated that by using liposome-polycation-hyaluronic acid (LPH) particles as a carrier for miRNA modified with a tumor targeting monoclonal antibody (GC4 single-chain variable fragment), they could target lung metastases in a murine model of metastatic melanoma [28].

3.2 Therapies that Restore miRNA Function

With regard to tumor suppressor miRNAs or those with decreased expression in benign disease states, the fundamental principle in miRNA-based treatment strategies is to restore their expression level to normal. This can be achieved through miRNA mimicry, viral vector-encoded miRNA replacement or by DNA demethylating agents.

3.2.1 miRNA Mimics

MiRNA mimics are small chemically altered double-stranded RNA molecules that imitate endogenous miRNAs [29], or precursor premiRNA molecules. Liu and coauthors observed that systemic delivery of miR-34a inhibited prostate cancer metastasis and extended survival of tumor-bearing mice, at least in part by targeting CD44 [30]. The delivery was achieved using intratumoral and, notably, intravenous injection and newly emerging delivery reagents such RNALancerII (BIOO Scientific) and siPORT™ amine (Ambion).

3.2.2 miRNA Genetic Precursors

Gene therapy in the form of viral vectors is another approach for the therapeutic replacement of miRNAs. Adenoviral and lentiviral vectors encoding miRNAs have been investigated as miRNA delivery vehicles in this context, with encouraging results [31, 32]. In fact, adenoviral vector-encoded miRNA replacement strategies have already been studied in vivo [33] and have attracted interest from miRNA therapeutics companies such as Mirna Therapeutics and Asuragen. These studies

reported transduction efficiency and minimal toxicity. However, Grimm et al. [34] highlighted the potential for serious toxicity to occur with this miRNA replacement strategy. Systemic administration of short RNAs was achieved in adult mice using a delivery vector based on duplex-DNA-containing adeno-associated virus type 8 (AAV8), resulting in down-regulation of critical liver derived miRNAs, resulting in morbidity and even fatality [34]. The authors postulated that mortality in this instance was consequent to oversaturation of endogenous miRNA pathways. Their experience is important to consider in bringing this strategy from bench to bedside. In parallel, another study demonstrated that systemic delivery of a miR-26a-expressing-adenoviral vector by intravenous injection resulted in impaired Myc-induced hepatic cancer progression by inducing tumor-specific cell cycle arrest and apoptosis [35].

A large body of evidence shows that most human tumors are characterized by defects in miRNA production that lead to global miRNA downregulation. It is therefore tempting to speculate that restoring the global miRNAome could have beneficial therapeutic effects. Global miRNA repression triggers cellular transformation and tumorigenesis in both *in vitro* and *in vivo* models [36–38]. As a result of these findings, a new 'miRNAome-based' strategy has been suggested. The small-molecule drug enoxacin enhances RNAi and promotes miRNA processing by binding to TARBP2 (Ref. [39]). Proof-of-principle studies in human cancer cell lines and xenografted primary tumors have shown that through global reconstitution of downregulated miRNAs to a more 'normal' miRNA expression pattern following enoxacin treatment, the malignant phenotype can be blocked [40]. The drug did not affect normal cells and was not associated with toxicity in mouse models [40].

3.2.3 DNA Demethylating Agents

Another approach for restoring the global miRNAome is the use of DNA demethylating agents and histone deacetylase inhibitors. These compounds reverse epigenetic silencing of tumor suppressor miRNAs and T-UCRs, thereby stopping tumor growth and ultimately resulting in the programmed cell death of the transformed cells [41–43]. These agents, even without any target specificity, have shown themselves to have therapeutic benefits and have received clinical approval for the treatment of certain haematological malignancies [44].

4 Delivery of Therapeutic MicroRNAs

Increasing evidence demonstrates that miRNAs are promising agents in cancer therapy. However, similar to other therapeutic oligonucleotides, the main challenge remains the successful delivery of therapeutic miRNAs to target tissues without compromising the integrity of the miRNA [45–47]. Naked ribonucleic acids are

subject to rapid nuclease dependent degradation and are therefore inherently unstable in biofluids. Thus, many therapeutic RNAi applications are limited to local administration where potential RNAi degradation is limited. However, local administration is only applicable to a short list of target tissues and frequently does not facilitate exposure of all diseased cells to the drug. Systemic delivery is therefore a better route of administration because—in theory—it provides a much more efficient dissemination of the therapeutic to target tissues. However, the miRNA will have to overcome many obstacles before it reaches the target. In addition, systemic delivery of miRNAs may induce similar adverse events that have been reported for other oligonucleotide-based therapies, such as aggregation and complement activation, liver toxicity and stimulation of an immune response by the nonspecific activation of toll-like receptors [48]. Criteria critical in the evaluation process are- (i) sufficient delivery to induce a therapeutic effect in disease models and (ii) a significant safety margin at therapeutic levels. Nevertheless, several strategies described above have had some measure of success, albeit in preclinical settings.

5 Dose Response and MicroRNA Therapeutics

For microRNA targeting therapeutics, there have been clear demonstrations of dose–response relationships in various animal models and microRNA targets. Using anti-miRs or antagomiRs to inhibit miR-122, multiple laboratories have demonstrated dose-dependent pharmacologic effects in species ranging from mouse to man [19, 20, 49, 50]. Thorough characterization of the dose response relationships *in vivo* may be complicated by the biology of microRNA/RNA induced silencing complex (RISC)-mediated effects in which the bottom portion of the typical sigmoidal curve may be very shallow owing to the relatively small changes in target de-repression observed even at maximal inhibition. Thus, dose response curves may take on the appearance of an all-or-none type of response simply because of the subtlety of the responses.

At the higher end of the dose response curve, like the more typical pharmacology of small molecule drugs, the pharmacologic effects of microRNA-targeting drugs should be saturable as the receptors become fully occupied. In the case of microRNA inhibitors, activity is dependent on the binding and sequestration of the target microRNA in an inactive microRNA/anti-miR heteroduplex. The receptor being in this case targeted microRNA. When all of the target microRNA in the cell is sequestered, addition of more anti-miR will not induce additional pharmacological effects but can induce non-specific ones. In any case, determining the saturating concentration or the dose of anti-miR required to produce saturation is critical in clinical trials where the desire is to produce the pharmacologic effect of interest at the lowest dose possible to avoid non-specific effects. For microRNA mimetics, in which the mature microRNA is replaced or expressed in a cell, the dose response relationships will be similar to those observed for exogenous siRNAs. Typical dose response relationships for exogenous siRNAs have been

reported in cell culture [51, 52] and in vivo in species from mouse to man [53]. The pharmacology of microRNA mimetics would be expected to be limited by the available RISC. Once these complexes have been completely occupied with the microRNA mimetic, the pharmacology would be expected to saturate. Additionally, there is the potential to induce unwanted effects when novel microRNAs are introduced into a cell. This problem can be avoided by administration of mimetics only for microRNAs that are ubiquitously expressed, thereby avoiding unwanted and unanticipated effects in a cell that has never expressed that microRNA, and only replacing activity needed to produce the pharmacology of interest in a target cell type that has lost the function of that microRNA. The field of microRNA mimetic toxicity is generally unexplored at this time. The potentially adverse effects of full saturation of RISC and other related complexes were demonstrated by the overexpression of an shRNA in mice [34] with lethal consequences. Whether exogenous microRNA mimetics can be delivered at concentrations that achieve those resulting from shRNA overexpression [34] has yet to be established, but these results suggest the nature of the effects of RISC saturation and other micro-RNA processing activities that could be extreme. A study by Khan et al. [54] might provide some insight into unexpected effects associated with RISC saturation. Khan et al. performed meta-analyses to conclude that siRNAs transfected into cells could compete with endogenous microRNAs for association with RISC, resulting in de-repression of micro-RNA targets [54]. Bioinformatic analyses of the transcriptome of tissues treated with miRNA mimetics will ultimately address these issues.

6 miRNAs in Renal Cancer

Renal cell carcinoma (RCC) is genetically and histopathologically a heterogeneous disorder. The most common subtype of RCC is clear cell RCC (ccRCC; approximately 75 %) and the next most frequent subtype is papillary RCC (pRCC; approximately 15 %) [55]. The most frequent genetic abnormality in ccRCC is inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene [56] and promoter methylation of tumor suppressor genes (TSGs) is common in both RCC subtypes. Dysregulation of miRNA expression is also pivotal for RCC development and progression. A number of miRNA expression and functional studies have been carried out in RCC. Table 10.1 presents a list of miRNAs that are either tumor suppressors or oncogenic in RCC. Our group has reported that several tumor suppressor and oncogenic miRNAs such as miR-1826 [57], miR-708 [58], miR-205 [59], miR-584 [60], miR-21 [61] are potential therapeutic targets in RCC. We observed that the expression of miR-205 was significantly suppressed in renal cancer cell lines and RCC samples [59]. This miRNA suppresses potential gene targets encoding Src, Lyn, Yes and Lck which are involved in cell migration invasion and cell proliferation. Therefore, its downregulation may facilitate RCC proliferation and diffusion. This study demonstrated that local administration of

Table 10.1 MicroRNAs and their targets in renal cell carcinoma

MicroRNAs	Function	Target gene	References
miR-99a	Tumor suppressor	mTOR	[64]
miR-138	Tumor suppressor	Vimentin	[65]
miR-204	Tumor suppressor	MAP1LC3B	[66]
miR-708	Tumor suppressor	Survivin	[58]
miR-1	Tumor suppressor	transgelin-2	[67]
miR-133a	Tumor suppressor	transgelin-2	[67]
miR-1826	Tumor suppressor	CTNNB1, MAP2K1	[57]
miR-34a	Tumor suppressor	c-Myc	[68]
miR-205	Tumor suppressor	Src kinase	[59]
miR-584	Tumor suppressor	ROCK1	[60]
miR-23b	Oncogenic	Proline oxidase	[69]
miR-21	Oncogenic	PTEN	[70]
miR-122	Oncogenic	PI3K/Akt	[71]
miR-30c	tumor suppressor	Slug	[72]
miR-590-5p	Oncogenic	PBRM1	[73]
miR-143/145	Tumor suppressor	hexokinase-2	[74]
miR-187	Tumor suppressor	B7 homolog	[75]
miR-1291	Tumor suppressor	Glucose Transportar 1	[76]
miR-1260b	Oncogenic	sFRP1, Dkk2, Smad4	[77]
miR-218	Tumor suppressor	CAV2	[78]
miR-30d	Tumor suppressor	MTDH	[79]
miR-21	Oncogenic	TCF21	[80]
miR-23b-3p	Oncogenic	PTEN	[81]
miR-135a	Tumor suppressor	c-myc	[82]
miR-99a	Tumor suppressor	Rapamycin	[64]
miR-210	Oncogenic	HIF1a	[83]
miR-101	Tumor suppressor	EZH2	[84]

miR-205 mimic complexed with SiPortAmine delivery reagent in established tumors induced a dramatic regression of tumor growth and hence miR-205 may be a potential therapeutic target for the treatment of RCC [59]. Another study by our group reported that miR-708 expression was widely attenuated in human RCC specimens. Restoration of miR-708 expression by miR-708 mimics in RCC cell lines decreased cell growth, clonability, invasion, and migration and elicited a dramatic increase in apoptosis. Moreover, intratumoral delivery of miR-708 mimic was sufficient to trigger in vivo regression of established tumors in murine xenograft models of human RCC. This report highlighted a major tumor suppressive role for miR-708 that may be an attractive target for therapeutic intervention in RCC [58]. Similarly, expression of miR-584 was found to be downregulated in RCC tissue samples [60]. It was correlated with high expression of R0CK-1 protein which modulates cell motility. This promotes tumor cell diffusion; therefore, targeting miR-584 may inhibit RCC progression and patient survival. Other laboratories have also studied numerous miRNAs as listed in Table 10.1, that might be therapeutic targets for the treatment of RCC. Aberrant expression of miR-1285 was

found to inhibit cancer cell proliferation, invasion and migration [62]. Downregulation of this miRNA, which targets oncogenic genes, might contribute to RCC development. This novel miRNA target may provide new insights into the potential mechanisms of RCC oncogenesis. Low levels of miR-508-3p and miR-509-3p have been found in plasma and renal cancer tissue samples of patients with RCC [63]. The over-expression of these miRNAs *in vitro* suppressed the proliferation of RCC cells, induced cell apoptosis and inhibited cell migration. These findings suggest that miR-508-3p and 509-3p play an important role as tumor formation modulators and may be novel RCC therapeutic targets. All these studies have reported gain or loss of function of individual miRNAs with pathological roles in tumor cell proliferation, progression and cancer development. These proof-of-principle studies in human cancer cell lines and primary tumor xenografts have demonstrated the therapeutic potential of targeting miRNAs for the treatment of renal cancer. Translation from an *in vitro*/local delivery to systemic *in vivo* delivery systems; however, remains a work in progress for RCC.

7 Closing Remarks and Conclusions

The global dysregulation of miRNAs has been described in several malignancies including cancers and it is clear that miRNAs can alter biological processes fundamental to tumor initiation and progression. miRNAs have quickly moved from discovery into therapeutic development programs. This rapid progress reflects the importance of miRNAs in cancer and is based on the thorough validation of key miRNAs as ideal candidates for therapeutic intervention. Although there is little doubt about their therapeutic potential, the challenge is to translate this potential into readily available drugs. The main focus in using miRNAs for cancer treatment is the problem of pharmacological delivery of miRNA, a task that has hampered the progress of related antisense and siRNA therapeutics. Yet, the recent clinical successes using existing delivery technologies and the continuous emergence of new delivery systems suggests that miRNA therapeutics for cancer is within the realm of possibility. As many current delivery systems show distinct biodistribution profiles, the type of cancer treated may largely depend on the performance of the underlying delivery system. Establishing ideal delivery systems with organ specificity while minimizing toxicity and off target effects will be essential to moving the field forward. It is envisioned that in the coming years there will be important advances in the field of miRNA-based therapeutics, and it remains to be seen whether these will result in effective miRNA-based-drugs.

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