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EXPERT REVIEW

The Therapeutic Potential of MicroRNAs: Disease Modulators and Drug Targets

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ABSTRACT MiRNAs are a class of small, naturally occurring RNA molecules that play critical roles in modulating numerous biological pathways by regulating gene expression. The knowledge that miRNA expression is dysregulated in many pathological disease processes, including cancer, has led to a rapidly expanding body of literature as we try to unveil their mechanism of action. Their putative role as oncogenes or tumour suppressor genes presents a wonderful opportunity to provide targeted cancer treatment strategies. Additionally, their documented function in a host of benign diseases broadens the potential market for miRNA-based therapeutics. The present review outlines the underlying rationales for considering mi(cro)RNAs as therapeutic agents or targets. We highlight the potential of manipulating miRNAs for the treatment of many common diseases, particularly cancers. Finally, we summarize the challenges that need to be overcome to fully harness the potential of miRNA-based therapies so they become the next generation of pharmaceutical products.

KEY WORDS miRNA manipulation · miRNA therapeutics · oncomirs · tumor supressors

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ABBREVIATIONS

AGO	argonaute
amiRNA	artificial microRNA
AML	acute myeloid leukemia
AMO	anti-miRNA oligonucleotide
ECM	extracellular matrix
ER	estrogen receptor
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HO-1	heme oxygenase-1
HSC	hepatic stellate cells
LNA	locked nucleic acids
MCL	mantle cell lymphoma
miRAGE	miRNA serial analysis of gene expression
miRISC	miRNA-associated RNA-induced silencing complex
miRNA	microRNA
mRNA	messenger RNA
NSCLC	non-small-cell lung cancer
PAMAM	polyamidoamine
PR	progesterone receptor
RAKE	RNA-primed array-based Klenow enzyme
SAGE	serial analysis of gene expression
SERM	selective estrogen receptor modulator
TGFß	transforming growth factor beta
UTR	untranslated region

INTRODUCTION

Mi(cro)RNAs are a class of small non-coding RNA fragments that have captured the attention of the scientific world since their discovery almost two decades ago. They have since been demonstrated to play critical roles in

almost all aspects of the cell cycle, and their expression is known to be dysregulated in various pathological conditions, including carcinogenesis (1). The functional roles of miRNAs in health and disease have been partly elucidated over the last 5 years; this process has unravelled their remarkable potential as disease biomarkers and therapeutic targets (2).

The association of aberrant miRNA expression with almost every cancer and common disease, along with functional analyses of specific miRNAs, has exposed the remarkable potential of manipulating miRNA expression as a therapeutic strategy for these conditions (Table I). The therapeutic application of miRNAs involves various strategies: first, through antisense-mediated inhibition of over-expressed miRNAs; second, through replacement of under-expressed miRNAs with either miRNA mimetics or viral vector-encoded miRNAs; and third, by modulating miRNA expression to augment a patient's response to existing treatment modalities (2).

MIRNA BIOGENESIS

The biogenesis of human miRNA originates in the nucleus, where there is transcription of a large primary (pri-)

Table I MiRNAs Implicated as Therapeutic Targets in Common Diseases

Disease	miRNA	Expression level in disease state	Stage of Investigation *(in vitro/in vivo)*
Hepatitis B virus	miR-122, miR-31	n/a	*In vivo* (21)
Hepatitis C virus	miR-122	n/a	*In vivo* (24,26)
	miR-199a	n/a	*In vitro* (29)
Hepatic fibrosis	miR-27a, miR-27b	Over-expressed	*In vitro* (30)
	miR-29a , miR-29b	Under-expressed	*In vivo* (31)
Hepatocellular carcinoma	miR-122	Under-expressed	*In vitro* (34–36)
Lung cancer (NSCLC)	Let 7 family	Under-expressed	*In vivo* (42–44)
	miR-21	Over-expressed	*In vivo* (47)
Pulmonary arterial hypertension	miR-204	Under-expressed	*In vivo* (48)
Breast cancer:			
Inhibition of metastases	miR-10b	Over-expressed[a]	*In vivo* (54)
	miR-21	Over-expressed	*In vivo* (55)
	miR-1258	Under-expressed	*In vitro* (56)
Breast Cancer:			
Response to adjuvant therapy	miR-21	Over-expressed	*In vivo* (58)
	miR-205	Under-expressed	*In vitro* (59)
	miR-128a	Over-expressed	*In vitro* (60)
	miR-125b	Over-expressed	*In vitro* (61)
	miR-155	Over-expressed	*In vitro* (62)
	miR-34a	Over-expressed	*In vitro* (63)
	miR-342	Under-expressed	*In vitro* (64)
Haematology:			
Leukaemia (B-CLL)	miR-15, miR-16	Under-expressed	*In vitro* (66)
AML	miR-29b	Under-expressed	*In vitro* (69)
Lymphoma	miR-17-92	Over-expressed	Tumour: *In vivo* (71)
	cluster		Radiotherapy: *In vitro* (72)
Prostate cancer	miR-34a	Under-expressed	*In vivo* (73)
	miR-16	Under-expressed	*In vivo* (74)
	miR-143	Under-expressed	*In vitro* (75)
Bladder cancer	miR-203	Under-expressed	*In vivo* (76)
Cardiac hypertrophy induced arrhythmia	miR-1, miR-133	Over-expressed	*In vivo* (77)
	miR-208	Over-expressed	*In vivo* (78)
	miR-100	Over-expressed	*In vitro* (79)
	miR-29	Under-expressed	*In vivo* (80)
Glioblastoma	miR-21	Over-expressed	*In vitro* (105)

[a] conflicting results reported by different studies

miRNA by RNA polymerase II or III. Seventy percent of human miRNAs are transcribed from introns and/or exons, suggesting that regulation of this process is under gene promoter control. The remaining 30% of pri-miRNAs are located in intergenic regions, and so have independent promoters (3,4). Pri-miRNAs are several hundred or thousand nucleotides in length and contain at least one miRNA stem loop. This single unit may contain up to six precursor (pre-) miRNAs, which are produced by the cleaving action of the RNase III enzyme Drosha, combined with the microprocessor complex subunit DGCR8. Pre-miRNAs range from 70 to 90 nucleotides in length and contain a hairpin structure that is critical for their transport to the cell cytoplasm by the energy-dependent Exportin-5 (5). Once in the cytoplasm this hairpin can then be cropped by the RNAase III enzyme Dicer, to produce a double-stranded structure, miRNA:miRNA*, consisting of the miRNA and its complement. This multi-step process culminates in the mature miRNA strand being incorporated into a miRNA-associated RNA-induced silencing complex (miRISC). It is in this formation that miRISC interacts with its target mRNA and exhibits its cellular effects (6) (Fig. 1).

MIRNA FUNCTION

MiRNAs have been implicated in almost every part of the cell cycle. They exhibit their function by sequence-specific modulation of gene expression at a post-transcriptional level. It is estimated that miRNAs govern over 30% of protein coding genes in this way (7). An understanding of their mechanism of action is crucial for their application in a therapeutic setting. The seed-sequence, the short region of importance in miRNA target recognition, extends from bases 2 to 8 on the 5′ tail of the mature miRNA strand (4). Each miRNA has two possible mechanisms of action, determined by the degree of complementarity between the miRNA seed sequence and its mRNA target, which is governed by Watson and Crick base pairing. First, if the target mRNA and miRISC have perfect base pairing

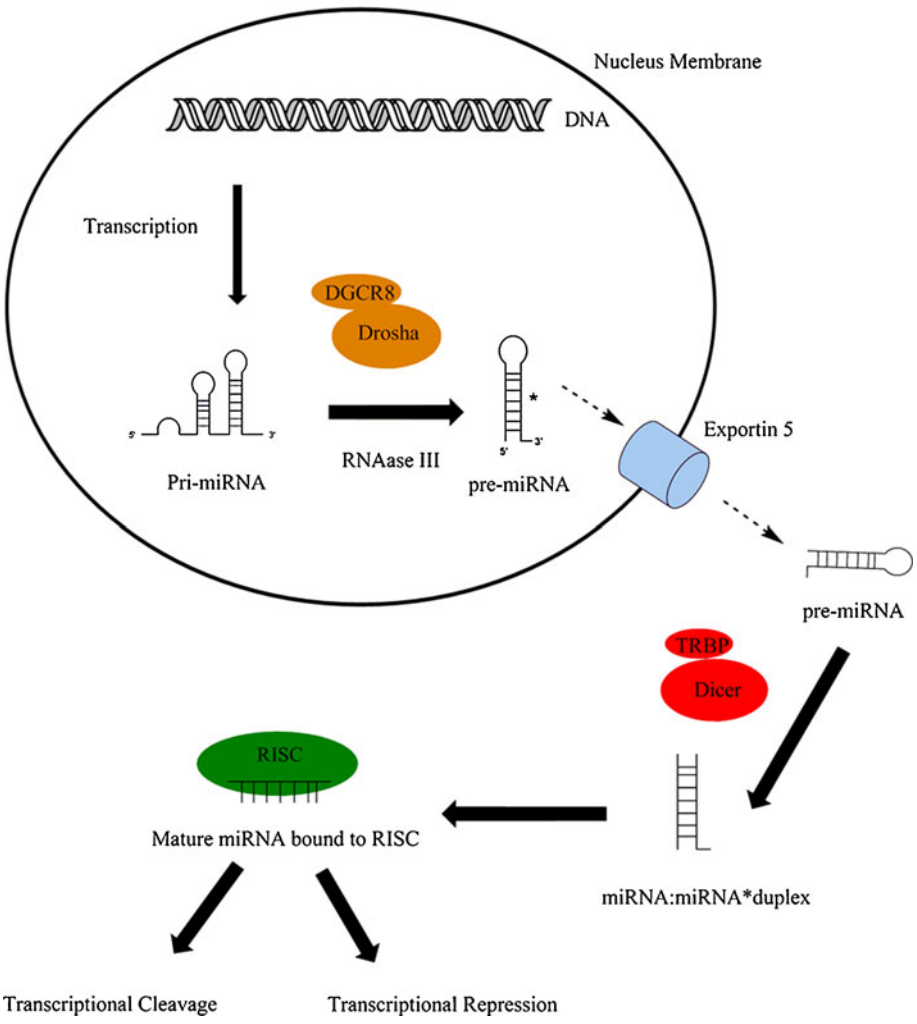

Fig. 1 MiRNA biogenesis and processing. Simplified representation of the steps involved in miRNA biogenesis and processing in human cells. This multi-step process begins in the nucleus of the cell, where there is transcription of a large primary (pri-) miRNA by RNA polymerase II. This large pri-miRNA is then cleaved by the RNase III enzyme Drosha and coupled with the microprocessor complex subunit DGCR8 to produce pre-miRNA. Pre-miRNAs range from 70 to 90 nucleotides in length and contain a stem loop structure for their transport to the cell cytoplasm by Exportin-5 (5). Once in the cytoplasm, this hairpin structure is cropped off by the RNase III enzyme, Dicer, producing the double-stranded miRNA:miRNA* duplex. This process culminates in the mature miRNA strand being incorporated into a miRNA-associated RNA-induced silencing complex (miRISC). It is in this formation that miRISC targets complementary mRNA sequences and exerts its cellular effects, via transcriptional cleavage or transcriptional repression.

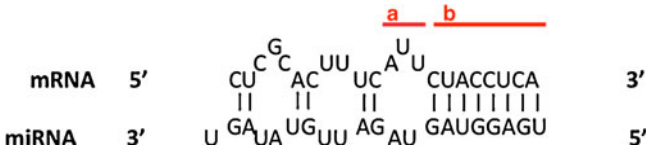

Fig. 2 Typical pattern of base pairing, with imperfect complementarity, between a miRNA and its target mRNA. Typically, the miRNA binds to a specific site or sites within the 3′UTR region of the mRNA sequence. According to thermodynamic analysis, some degree of complex formation occurs along the entire miRNA-mRNA duplexed region. Base pairing is particularly weak in the central region due to mismatched 'bulges' in the miRNA sequence (**a**), and particularly strong at the 5′ end (seed region) of the miRNA (**b**). Base pairing between *let-7* miRNA and *hbl-1* mRNA in *C. elegans* is shown as an example (Lin *et al.*, 2003).

homology, the mRNA is cleaved and degraded through activation of the RNA-mediated interference pathway. Second, and more commonly, miRNAs modulate their gene targets by repression of protein translation. MiRNAs exhibit this effect by imperfectly binding to partially complementary sequences located often in the 3′ untranslated region (UTR) of target mRNAs, although miRNAs can also bind to the coding region and 5′UTR of target genes (8). The proposed mechanism by which imperfect pairing between a miRNA and its target results in translation inhibition or repression is that efficiency of translation is reduced consequent to various mismatched 'bulges' in the central region, or to a lesser extent the 3′end, of the miRNA (Fig. 2). These bulges appear to affect the strength with which the miRNA binds to its mRNA target and can affect the Argonaute (AGO)-mediated endonucleolytic cleavage of mRNA (9). Thus far, over 1,000 human miRNAs have been identified (10), each with the capacity to influence several mRNA targets through imperfect base pair homology (Fig. 3).

MIRNA PROFILING AND IDENTIFICATION OF DISEASE-SPECIFIC MIRNAS

MiRNA expression profiling of a variety of human tissues, both healthy and pathological, has given remarkable insight into the developmental stages of many diseases. It has been shown that distinct patterns of miRNA expression are observed in individual tissues and in different disease states. These tissue- and disease-specific expression patterns reflect mechanisms of cellular transformation and further support the idea that miRNA expression patterns encode the developmental history of human disease. In contrast to mRNA expression profiles, it is even possible to successfully classify poorly differentiated tumors using miRNA expression patterns (11,12). A number of different techniques are available for miRNA expression profiling. Oligonucleotide microarray-based miRNA analysis was first described in 2004 and has since become the most commonly used method for detecting cancer-specific miRNA expression profiles involving large numbers of samples (13). Bead-based flow cytometric technology is a highly specific high-throughput method of miRNA expression profiling, devel-

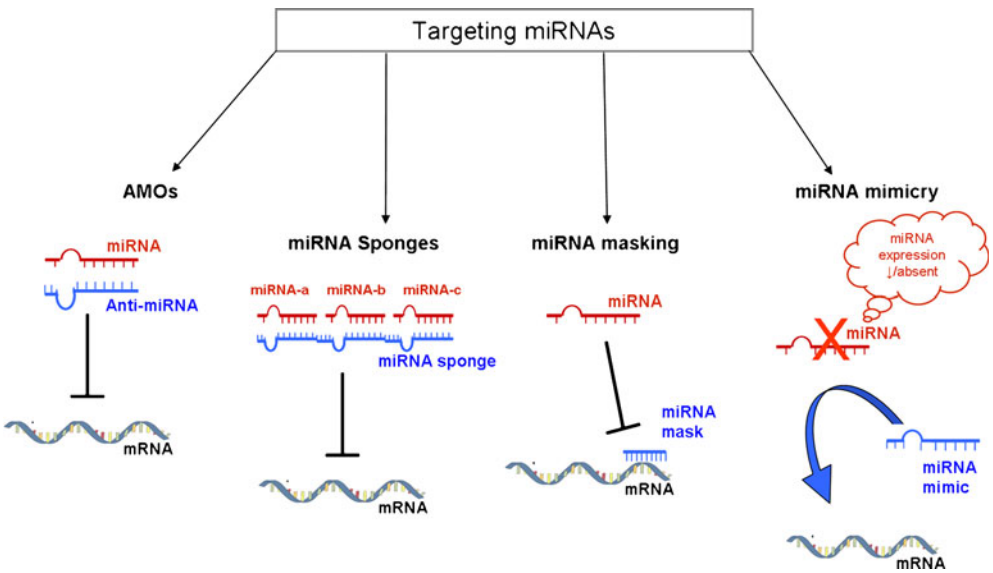

Fig. 3 Strategies of miRNA manipulation and potential miRNA therapeutic strategies. The effects of oncogenic miRNAs can be down-regulated by anti-miRNA AMOs (anti-miRNA oligonucleotide), miRNA sponges, and miRNA-masking. AMOs can bind to complementary miRNAs and induce either duplex formation or miRNA degradation. MiRNA sponges exhibit multiple miRNA binding sites, resulting in the ability to simultaneously sequester multiple miRNAs. MiRNA masks are complementary to the 3′UTR of the target miRNA, resulting in competitive inhibition of the downstream target effects. The downstream effects of tumour suppressor miRNAs can be restored by introducing synthetic miRNAs (miRNA mimicry).

oped by Lu *et al.* (12). While microarray-based miRNA profiling experiments are technically more challenging to perform, bead-based flow cytometry provides a higher specificity. Other technologies in this realm include tag-based sequencing methods such as miRNA serial analysis of gene expression (miRAGE) (14) and the high-throughput RNA-primed array-based Klenow enzyme (RAKE) assay, which is an enzymatic on-chip-labeling technique (15). However, laborious and costly cloning and sequencing steps have limited the use of SAGE, and widespread use of the RAKE assay has been hindered primarily by the fact that a large amount of starting RNA is required (16). More recently, the introduction of platforms that permit large-scale parallel analysis of genome-wide sequences have advanced miRNA identification and analysis even further. Deep sequencing technology is one such platform which enables the simultaneous sequencing of millions of different RNA molecules in a single sample. Deep sequencing overcomes many of the limitations of microarray-based profiling. The latter is susceptible to cross-hybridization and measures only the relative abundance of miRNAs that have already been identified. In contrast, deep sequencing is not dependent on any prior sequence information. Instead, it provides unbiased information about all RNA species in a given sample, thus allowing for discovery of novel and disease-specific miRNAs or other types of small RNAs that have eluded previous cloning and standard sequencing efforts. In conjunction with the evolution of next-generation sequencing technologies, advanced bioinformatic tools have had to evolve simultaneously in order to analyze the massive amounts of data generated (17–20). As these highly sophisticated techniques continue to develop, the extent and significance of miRNA regulation of gene expression will become even more evident. The future of miRNA expression profiling may lie in techniques which can be applied to profile miRNA expression *in vivo*, and not just in archived specimens. Molecular imaging of miRNAs presents a non-invasive method of monitoring miRNA biogenesis and function based on reporter and fluorescent beacon imaging approaches. Molecular imaging is superior to traditional miRNA expression profiling methods, as it can be applied to living cells and provides further insight into potential disease altering miRNAs for consideration in therapeutic modalities (21). This exciting development could be invaluable in the clinical setting, allowing individual response to treatment to be evaluated at a cellular level.

MIRNAS AND THERAPEUTICS

The rapidly expanding body of knowledge on miRNA expression and function is ideal for exploiting as the next generation of disease therapeutics. The fact that these tiny RNA fragments are implicitly involved in many pathological states and that they mediate potent and specific gene silencing makes them attractive therapeutic targets. To date, the greatest efforts in this setting have been in exploring the potential application of miRNA therapeutics for various cancers. In the cancer state, miRNAs have been demonstrated to play a dual role, that of an oncogene or a tumor suppressor. Gain or loss of function of individual miRNAs has been reported in almost every solid and hematological cancer, with pathological roles in tumor cell proliferation, progression of tumors and the metastatic process (1,22). Early *in vitro* work involving miRNA manipulation in cancer cell lines demonstrated the remarkable therapeutic potential of this strategy. A number of different molecular and pharmacological strategies may be employed to help realize this potential.

MiRNAs with oncogenic capacity can be deactivated or silenced by several RNA interference-type strategies, namely miRNA-specific knockdown by anti-miRNA oligonucleotides (AMOs), miRNA sponges and miRNA masking. AMOs are synthetic antisense oligonucleotides that competitively inhibit the interaction between miRNAs and their mRNA targets. The most widely employed types of AMOs are 2′-O-methyl AMOs, 2′-O-methoxyethyl AMOs and locked nucleic acids (LNAs) (23). Locked nucleic acids (LNA) are modified oligonucleotides with many advantages over traditional AMOs, including the fact that they do not require a vector and have superior thermal stability and lower toxicity (24). These latter molecules are being utilized in the majority of current *in vivo* studies in this field. As a potential therapeutic approach, however, AMOs have several inherent weaknesses, such as their transient duration of action and inability to target more than one miRNA at a time.

Given that miRNAs have been observed to function not in isolation but often in clusters in pathological processes, knockdown of multiple over-expressed miRNAs presents a therapeutic challenge. The unique concept of ‘miRNA sponges’ holds great appeal in this context. These competitive miRNA inhibitors are transcripts expressed from strong promoters that display numerous and tandem binding sites for the miRNAs of interest. Sponges, which may be located in non-protein coding RNA or in the 3′-UTR of a reporter gene, are frequently under the control of potent promoters, such as CMV, to ensure large quantities of the transcript are produced (25). Ebert *et al.* demonstrated the efficacy of these miRNA inhibitors *in vitro* by transiently transfecting cultured cells with vectors encoding miRNA sponges. This resulted in a reduction in the level of miRNA targets to at least that attainable with AMOs (12,26). A single sponge bearing a heptameric seed sequence can target families of over-expressed miRNAs which share this seed. In doing so, the sponge can effectively manipulate abnormal expression levels, thereby

preventing their binding with endogenous mRNA targets (26). Drosphilia miR-SP is a dynamic technology that allows transgenic miRNA silencing, with precise *in vivo* spatial resolution (27). This advanced miRNA-sponge technology aims to overcome the lack of tissue specificity associated with traditional miRNA-sponges, while providing insight into interactions between miRNAs and other genes. Transgenic miRNA sponges (miR-SPs) are synthesised by locating modified miRNA complementary oligonucleotides downstream of repetitive upstream activation sequences (UAS).

MiRNA masking is an alternative miRNA knockdown strategy to the AMO approach, with the advantage of targeting miRNAs in a gene-specific manner (28). A miR-mask is synthesized as a single-stranded 2′-O-methyl-modified oligoribonucleotide, which has perfect complementarity to an endogenous miRNA binding site in the 3′ UTR of a protein coding mRNA gene. Unlike an AMO, which binds to the target miRNA directly, a miR-mask binds with high affinity to the target miRNA's binding site in the 3'UTR of its mRNA target. This specific mechanism avoids off-target effects. The miR-mask technology has already been validated *in vivo*, thereby highlighting its potential clinical utility. Using a zebrafish model, Choi *et al.* successfully inhibited the repressive action of *miR-430* on transforming growth factor beta (TGFß) using a miR-mask, which was complementary to the *miR-430* binding site in its target mRNAs *squint* (*sqt*) and *lft2* (29).

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 or viral vector-encoded miRNA replacement. MiRNA mimics are small chemically altered double-stranded RNA molecules that imitate endogenous miRNAs (30), or the precursor pre-miRNA molecules. The viability of this approach has been demonstrated in numerous *in vitro* and *in vivo* settings, the details of which will be discussed later in this review. 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.* 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 fatalities (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. We will now discuss the rationale and evidence for miRNA therapeutic applications in many common diseases.

LIVER DISEASES

The seminal advances with respect to miRNA therapeutics have been in the field of liver disorders: hepatitis, hepatic fibrosis, and hepatocellular carcinoma (HCC). HCC is one of the most common cancers worldwide and among the leading causes of cancer-related deaths (35). It usually arises in the setting of pre-existing chronic liver disease, which is caused by viral hepatitis (B or C) in 80% of cases worldwide (36). The role of miRNAs in viral hepatic diseases is particularly complex. In addition to miRNA-mediated RNA-silencing pathways influencing viral-host cell interactions (37), viruses not only exploit the hosts cellular miRNAs, but also encode their own miRNAs (38).

Viral Hepatitis B

There is compelling evidence to suggest that miRNAs participate in the development of and host reponse to hepatitis B viral infection (39). Using computational analysis, Jin *et al.* identified that HBV putatively encodes only one candidate pre-miRNA and that viral miRNA only targeted viral mRNA, not host cellular transcripts. The authors proposed that HBV had evolved to use viral miRNAs as a means to regulate its own gene expression to its benefit (40). This hypothesis was confirmed *in vitro* when vector-based artificial miRNA (amiRNA) successfully inhibited HBV replication and expression (41). Ely *et al.* confirmed the *in vivo* viability of this potential therapeutic approach to HBV by employing RNA polymerase II promoter cassettes that transcribes anti-HBV primary miRNA shuttles, specifically pri-mi-122 and pri-mi-31, with a resulting decreases in HBV expression (42).

Viral Hepatitis C

Relative to HBV, there is less evidence to support the involvement of viral miRNAs in the replication of hepatitis C virus (HCV). However, HCV replication appears to be subject to the regulatory miRNAs of the human host cell (39). *MiR-122* was the first liver-specific cellular miRNA identified and constitutes over 70% of miRNAs in the liver. It is known to have two potential binding sites for HCV and enhances the replication of HCV by targeting the viral 5′

non-coding region. Within hepatic tissue, *miR-122* is only detected in the HuH-7 human hepatoma cell line, which is interesting, as HCV can only replicate in these cells. When *miR-122* is inactivated *in vitro* by transfection with 2′-O-methylated RNA oligonucleotide with exact complementarity to *miR-122*, HCV replication in these cells decreases by over 80% (43). This confirms that *miR-122* plays an important direct role in HCV translation by targeting the 5′ untranslated region and enhancing the association of ribosomes at an early stage (44). Krutzfeld *et al.* provided the first report of successful miRNA antagonism *in vivo* when *antagomir-122* was conjugated with cholesterol and delivered intravenously, resulting in *miR-122* knockdown for 23 days (45). However, this method of antagmiR delivery, employing synthetic 2′-O-methyl anti-miRNA oligonucleotides (AMOs), raises concerns regarding their stability and toxicity. Locked nucleic acid (LNA)-modified oligonucleotides, as mentioned, present significant advantages for sequence-specific antagonism of miRNAs; they display advanced thermal stability when combined with their target RNA and have a low toxicity profile in mammals (24,46). The use of LNAs *in vivo* as a mechanism of delivering miRNAs for therapeutic purposes was demonstrated by Elmen *et al.* In a murine model, this group delivered unconjugated LNA-antimiR oligonucleotide complementary to the 5′end of *miR-122*, and observed specific dose-dependent *miR-122* silencing without hepatotoxicity (47). It has also been documented that *miR-122* is an indirect facilitator of HCV replication; Heme Oxygenase-1 (HO-1) is capable of inhibiting HCV replication, and *miR-122* down-regulates this pathway. The combination of *miR-122* down-regulation, with up-regulation of HO-1, is a potential new strategy for antiviral therapies directed towards HCV (48).

MiR-199a is another liver-specific miRNA that has been associated with HCV replication. *In vitro* studies have demonstrated that over-expression of *miR-199a* results in inhibition of HCV replication, independent of the interferon pathway, while inactivation of *miR-199a* induces accelerated viral replication (49).

Hepatic Fibrosis

Liver fibrosis is a largely irreversible condition that occurs in association with most chronic liver diseases. Hepatic stellate cells (HSCs) become activated in response to repeated injury and exposure to inflammatory mediators. They subsequently lose their lipid droplets and migrate to the injured area, where they secrete large amounts of extracellular matrix (ECM), resulting in fibrosis (50). This process can result from chronic hepatitis, and ultimately leads to liver cirrhosis and potentially hepatocellular carcinoma. Many miRNAs have been implicated in the pathogenesis of hepatic fibrosis. *MiR-27a* and *miR-27b* have recently been studied in rat HSCs *in vitro*. They are normally over-expressed in the inflammatory state, and down-regulation of both miRNAs resulted in the HSCs returning to a more quiescent state, with decreased proliferation and restored lipid droplets (51). *MiR-29a* and *miR-29b* are also of interest in the setting of hepatic fibrosis. A recent microarray conducted on murine livers identified the *miR-29* family as being significantly down-regulated in fibrotic liver tissue (52). In this elegant study by Roderburg *et al.*, *miR-29* was shown to play a regulatory role in pathways involving the genes TGF-β and NF-κβ. The authors also found that over-expression of *miR-29b* resulted in down-regulation of collagen expression in murine HSCs (52). These data illustrate the future potential for *miR-29b* as an antifibrotic agent.

Hepatocellular Carcinoma

MiR-122 is one of the most extensively investigated miRNAs; it is now known that its function extends far beyond virus replication and infection of the liver. Computational tools and *in vitro* expression data suggest that *miR-122* also has a role in the cellular stress response (53) and hepatocellular carcinogenesis (54). Converse to the major positive role of *miR-122* in HCV replication, it has a negative role in hepatic tumorigenesis and in fact is a tumor suppressor in the liver. MiRNA expression profiling has revealed that *miR-122* is down-regulated by at least 50% in human HCC tissue compared to normal or non-cirrhotic liver.

Transfection of HCC cell lines with *miR-122* has been shown to induce cellular apoptosis and reduce cell viability (55,56). This presents a novel chemotherapeutic strategy in HCC, a disease with a typically poor prognosis for which there are limited treatment options. An increase in *miR-122* expression in malignant cells could result in targeted cell death. Young *et al.* devised a mechanism to test this theory by developing small molecule modifiers of *miR-122* function (57). These miRNA modifiers (1–3) act at the transcriptional level to either up- or down-regulate *miR-122* expression. More specifically, the authors observed that small molecule *miR-122* inhibitor 2 inhibited HCV replication, while small molecule *miR-122* inhibitor 3 induced an increased expression of the pro-apoptotic *miR-122* in the HCC cell line HepG2, leading to *caspase* activation and reduced cell viability. This study highlights the remarkable potential of miRNA manipulation as a plausible novel therapeutic strategy.

LUNG DISEASES

Lung cancer is the leading cause of cancer death worldwide, with non-small-cell lung cancer (NSCLC) accounting

for 80–85% of lung cancer cases. To date, over 40 miRNAs are known to be dysregulated in NSCLC. Various miRNA signatures, derived from lung tumor tissue or even plasma/serum samples, have been proposed as biomarkers of this disease with utility in diagnosis and prediction of overall survival (58,59). With regard to miRNA therapeutic strategies for lung cancer, much of the work to date has focused on harnessing the tumor suppressor properties of the *let-7* family for this purpose. *Let-7* is stably expressed in normal adult lung; however, expression profiling of NSCLCs has revealed that various members of the *let-7* family are decreased in tumor tissue compared to normal lung. *Let-7a*, among other miRNAs, has been shown to have prognostic value in that low levels correlate with poor survival (60). Functional work has identified and defined the regulatory roles of the *Let-7* family in several oncogenic pathways, including the RAS pathway, where it represses activity of the *KRAS* oncogene, mutations of which are commonly implicated in adenocarcinoma of the lung (61,62).

Esquela-Kerscher and Slack *et al.* pioneered many of the early *in vitro* and *in vivo* investigations into the role of let-7 miRNAs in NSCLC. They identified that the tumor suppressor effect of *let-7* was transient and that replacement of let-7 through gain-of-function techniques could reduce cell proliferation in various human lung cancer cell lines. In a murine model of human lung cancer, this group demonstrated how loss of *let-7* induced lung tumor formation and growth, through loss of its regulatory effect on the oncogenes *RAS* and *HMGA2* amongst others. Restoration of *let-7* expression in lung cancers, using intranasal delivery techniques, restrained the growth of tumors by repressing multiple cell cycle and proliferation pathways together with *ras* and *MYC* suppression (63,64). This work paved the way for further investigations into the therapeutic feasibility of miRNAs in the clinical treatment of lung cancer.

Although promising, the application of *let-7* as a therapeutic agent for cancer is premature as yet, given that details of the immunogenic and cytotoxic effect of *let-7* administration remain to be determined. Its ubiquitous expression and involvement in multiple cellular pathways imply that manipulation of its levels is likely to have diverse off-target effects. The development of safe, effective, and tissue-specific delivery methods for *let-7* requires further effort before this strategy advances as a cancer therapy.

Kumar *et al.* have demonstrated similar tumor suppressor effects of the *let-7* miRNAs on lung cancers *in vivo* (65). Using a lentiviral system, they first transfected murine *KRAS*-expressing lung adenocarcinoma cells (LKR 13) with a *let-7 g* miRNA duplex; this resulted in decreased cell proliferation and induction of cell death. In tumor xenografts, the authors observed significant reduction of both murine and human non-small-cell lung tumors when *let-7 g* was over-expressed using these lentiviral vectors. Furthermore, they found that *let-7 g*-mediated tumor suppression was more pronounced in lung cancer cell lines harboring oncogenic K-Ras mutations than in lines with other mutations. The potential of *let-7* in the treatment of lung cancer extends beyond its direct effects on the tumor. Preliminary *in vitro* data suggest that there is potential to use miRNA modulation to enhance standard treatments for cancer, such as radiotherapy for lung tumors. Weidhaas *et al.* provide evidence that over-expressing members of the *let-7* family in lung cancer cells and in a *C. elegans* model of radiation-induced cell death results in increased sensitivity to radiation therapy, whereas decreasing *let-7* levels induces a state of radioresistance (66). These effects were mediated through altered *RAS* signalling.

The potential role of miRNAs in treating lung disorders is not confined to *let-7*. Blower *et al.* showed that altering expression levels of *let-7i*, *miR-16* and *miR-21* in a lung cancer cell line (A549) altered the potency of chemotherapeutic agents up to four-fold (67). Manipulation of the oncogenic *miR-21* in NSCLC has also been investigated as a possible therapeutic strategy. Using transgenic mice, Hatley *et al.* demonstrated that over-expression of *miR-21* was associated with cell proliferation and tumor growth, whilst genetic deletion of *miR-21* partially protected against tumor formation (68). Inhibiting *miR-21* increased tumor sensitivity to DNA-damaging chemotherapeutic agents and could potentially restore the activity of multiple tumor suppressors acting at various critical points of tumorigenesis.

Pulmonary arterial hypertension has also been associated with aberrant miRNA expression and function. Decreased *miR-204* levels in affected lungs have been shown to correlate with disease severity in both animal and human studies (69). Targeted delivery of synthetic *miR-204* to the lungs of affected animals resulted in a significant reduction in disease severity. This illustrates another potential application of miRNA therapeutics.

BREAST CANCER

Since Iorio *et al.* first reported dysregulated miRNA expression in breast tumors in 2005, evidence has accumulated implicating miRNAs as key players in breast tumorigenesis, progression, and metastases and in determining tumor response to existing treatments (70,71). As in other cancers, miRNAs play dual roles as oncogenes or tumor suppressors in this prevalent disease. In a therapeutic capacity, there have been two predominant objectives and approaches to manipulating miRNA expression in breast tumors thus far: knockdown of candidate breast cancer-related 'oncomirs' to suppress tumor growth and inhibit or prevent distant metastases, and modulation of miRNA expression with the

intent of augmenting or altering tumor responsiveness to adjuvant chemotherapeutic or hormonal agents.

'OncomiR' Knockdown

It is widely accepted that metastases are responsible for most cancer-related deaths. However, targeting or interrupting the metastatic process with therapeutics has been largely unsuccessful as a result of our limited understanding of this pathological process (72). Recent endeavours to explore the role of miRNAs in the metastatic cascade have identified potentially key pathways in this process and novel therapeutic targets. Accumulating data have proven that miRNAs exert their effects at multiple steps in the metastatic cascade by influencing cancer cell adherence, migration, invasion, motility, and angiogenesis (73). In their miRNA microarray analysis of paired tumor tissues and metastatic lymph nodes, Baffa *et al.* identified a metastatic cancer miRNA signature inclusive of *miR-10b, miR-21, miR-30a, miR-30e, miR-125b, miR-141, miR-200b, miR-200c,* and *miR-205*. *MiR-10b* is implicated in many cancers, including breast cancer, and is thought to promote tumor invasion and metastasis by inhibiting translation of the *HOXD10* gene, thereby resulting in increased expression of the pro-metastatic gene, *RHOC* (74). Weinberg's group has also recently reported exciting findings from work on antagonizing *miR-10b* in metastatic breast cancer cell lines (MDA-MB-231 cells). Silencing *miR-10b* with antisense oligonucleotides was found to inhibit *Twist*-mediated cell migration and invasion. They observed similar anti-metastatic effects after systemic *miR-10b* antagonism in a murine model (75).

Numerous other miRNAs have been implicated in the metastatic pathway. Yan *et al.* performed *in vitro* LNA silencing of *miR-21* in two breast cancer cell lines (MCF-7 and MDA-MB-231), which resulted in significantly reduced cell proliferation and migration. Their subsequent *in vivo* studies resulted in similar inhibition of breast tumor growth following *miR-21* knockdown with antimiRs (76). Zhang *et al.* have been first to report that *miR-1258* inhibits breast cancer brain metastases by negatively regulating the heparanase pathway (77). Again, these results strongly support the potential of miRNAs to be applied to the clinical setting for therapeutic gain.

Augmenting Response to Adjuvant Therapy

Chemotherapeutic drugs, radiotherapy and endocrine agents (aromatase inhibitors and selective oestrogen receptor modulators, SERMs) are the adjuvant therapies used in the routine management of women with breast cancer at present. Despite their success in improving disease-free and/or overall survival, a proportion of women derive no benefit from these treatments or develop resistance to these agents over time (78). The basal subtype of breast cancer (classically ER, PR and HER2/*neu* negative) presents a specific therapeutic challenge, as there are no targeted therapies currently available. Preliminary studies suggest that miRNA modulation in tumor tissue can augment its response to systemic therapies. *MiR-21* is again one of the most studied miRNAs in this setting. Mei *et al.* combined taxol chemotherapy with *miR-21* inhibitor treatment, via a polyamidoamine (PAMAM) dendrimer vector, to evaluate the effects of combination therapy on suppression of breast cancer cells and found that cells treated with this combination demonstrated significantly reduced cell viability and invasiveness compared with cells treated with taxol alone, reflecting an enhanced chemotherapeutic effect of taxol in the presence of decreased *miR-21* levels (79).

There is also experimental evidence that manipulation of *miR-205* levels can improve breast tumors' response to anticancer agents. Based on computational target prediction algorithms, Iorio *et al.* hypothesised that *miR-205* was involved in regulation of the HER3 receptor, a kinase-inactive member of the HER family which plays an important and necessary function in HER2-mediated tumorigenesis. Indeed, their *in vitro* experiments demonstrated that *miR-205*, which is down-regulated in breast tumors compared with normal breast tissue, directly targeted the HER3 receptor and inhibited activation of the downstream mediator Akt (80). Furthermore, reintroduction of *miR-205* in SKBr3 cells was found to inhibit their clonogenic potential and increase the responsiveness of these cells to the tyrosine-kinase inhibitors Gefitinib and Lapatinib, thus overcoming HER3-mediated resistance and restoring proapoptotic activity.

Manipulation of several other miRNAs has been shown to have the potential to augment breast tumors' responsiveness to existing therapies. Inhibition of endogenous *miR-128a*, which is highly expressed in letrozole-resistant breast tumors, overcomes resistance to the aromatase inhibitor letrozole by modulating TGFß signalling (81). *MiR-125b, miR-155,* and *miR-342* have also been implicated in regulating chemosensitivity, whilst knockdown of *miR-34a* is associated with increasing cancer cells sensitivity to radiation (82–85).

HEMATOLOGY

Much of the initial data on miRNA expression profiling and function stemmed from studies of hematological malignancies. However, few experiments testing the therapeutic potential of miRNAs in this setting have been conducted.

Leukemia

The first evidence of involvement of miRNAs in malignancy came from the identification of a translocation-induced

deletion at chromosome 13q14.3 in B-cell chronic lymphocytic leukemia (86). Loss of *miR-15a* and *miR-16-1* from this locus results in increased expression of the anti-apoptotic gene *BCL2* (87). *BCL2* inhibition through replacement of these deficient miRNAs is therefore a plausible therapeutic strategy. Similar potential exists for miRNA-based therapeutics in the management of acute myeloid leukemia (AML), for which distinctive patterns of aberrant miRNA expression have been identified (88). Eyholzer *et al.* demonstrated that *miR-29b* expression is decreased in AML patients displaying either CEBPA deficiency or loss of chromosome 7q (89). Data from Calin and Croce's laboratory showed that restoration of *miR-29b* in AML cell lines and primary bone marrow or peripheral blood samples induced apoptosis and dramatically reduced tumorigenicity in a xenograft leukemia model (90).

Lymphoma

The *miR-17-92* cluster is located in a region frequently amplified in B-cell lymphoma (91). This cluster, located at chromosome 13q31-q32, is comprised of seven individual miRNAs that are transcribed as a polycistronic unit (*miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-19b, miR-20a,* and *miR-92*). He *et al.* clearly illustrated the oncogenic activity *of miR-17-92,* by demonstrating accelerated tumor development and reduced Myc-induced apoptosis in a Eu-Myc transgenic mouse model of human B-cell lymphoma (92). In theory, modulating this cluster of miRNAs could inhibit lymphoma progression. Targeting the *miR-17-92* cluster could also be used to augment response to radiotherapy in human mantle cell lymphoma (MCL). MCL cells over-expressing *miR-17-92* display increased cell survival and reduced cell death following radiotherapy. Knockdown of this miRNA cluster could increase the radiosensitivity of MCL cells, thereby improving prognosis for these patients (93). The miRNA sponge concept would be an ideal therapeutic strategy in this setting, as a single sponge molecule could target the entire miRNA cluster simultaneously.

MIRNA THERAPEUTICS IN OTHER COMMON CONDITIONS

In addition to cancers, miRNAs are known to be dysregulated in a wide range of other disease processes. From a therapeutic perspective, the most promising applications at present for miRNA-based treatments are in the settings of urological, cardiovascular and neurological diseases.

MiR-34a is known to be under-expressed in prostate cancer cells, which exhibit advanced proliferation and metastatic potential and express the adhesion molecule CD44. Liu *et al.* demonstrated that increasing the expression of *miR-34a* in these CD44(+) prostate cancer cells suppressed tumor progression and metastases and resulted in increased survival in a mouse model. Furthermore, inhibition of *miR-34a* in CD44(−) prostate cancer cells by administering *miR-34a* antagomiR contributed to increased tumor burden and metastases (94). This study provides evidence to support the suggestion that the negative regulatory effect of *miR-34a* on CD44 could be exploited for therapeutic benefits in prostate cancer. Several other miRNAs have been implicated as inhibitors of the metastatic process in prostate cancer, including *miR-16* and *miR-143* (95,96). Bladder cancer is also associated with aberrant expression of miRNAs, such as *miR-203,* which represent ideal therapeutic targets. *In vitro* data have identified pro-apoptotic effects of *miR-203* on bladder cancer cells through its down-regulatory effect on bcl-w, implying that gain-of-function modulation with *miR-203* mimetics has potential utility in the treatment of this malignancy (97).

Altered miRNA expression has also been demonstrated in various cardiovascular diseases, including heart failure, arrhythmias, and fibrosis, unveiling further opportunities for miRNA-targeted therapies. Cardiac hypertrophy and its associated arrhythmias may be suppressed by *miR-1* and *miR-133* over-expression through post-transcriptional repression of HCN2 and HCN4 genes (98). *MiR-208a* and *miR-100* have also been implicated as a modulator of cardiac hypertrophy and electrical conduction (99,100). Myocardial infarction can be complicated by fibrin deposition in the damaged muscular wall, the adverse consequences of which include stiffening of the ventricular walls, diminished contractility, and abnormalities in cardiac conductance. Increasing the expression of *miR-29b* in cardiac fibroblasts has been shown to decrease the expression of collagen transcripts in these cells, hence reducing collagen production (101). This knowledge highlights *miR-29b* as a potential therapeutic agent for fibrotic diseases.

An important role of miRNAs in neurological conditions has also been identified. This work has stimulated the expectation that miRNAs hold potential as therapeutic agents for the treatment of debilitating neurodegenerative conditions such as Huntington's, Parkinson's, and Alzheimer's diseases, for which no disease-modifying treatment strategies exist currently (102). Animal model studies have shown that loss of neural miRNAs may be involved in the development and progression of these neurodegenerative diseases. *In vitro* experiments provide further support for miRNAs as therapeutic agents in these conditions; they have been shown to partially preserve miRNA-deficient neurons when over-expressed in these cells. A serious limitation of many novel drugs in this setting to date has been their inability to cross the blood-

brain barrier. Indeed, this will remain one of the major challenges in developing miRNA-based therapeutic strategies for neurological diseases.

POTENTIAL CHALLENGES

The rush to identify novel miRNAs with a role in specific disease processes continues in an effort to expedite the transition of miRNA-based therapeutics from bench to bedside. While significant advances have been made in this field to date, various challenges remain to be overcome before miRNA therapies become a reality. The development of therapeutic strategies involving disease-specific miRNAs is subject to identification and validation of their multiple mRNA targets and to elucidating the complex pathways which they partly or wholly regulate. A significant amount of functional work remains to be performed in order to achieve this. Thus far, predicting gene targets of miRNAs has been largely computationally governed, and miRNA targets are predicted by sequence complementarity rather than in a gene-specific manner. This complex approach to target identification, along with the fact that individual miRNAs have multiple potential targets, leads to difficulty in predicting the spectrum of side effects and toxicity profiles which may be associated with miRNA-based therapeutics. Only *in vivo* investigations followed by carefully designed early phase clinical studies will identify these issues and help overcome them.

Another obstacle which must be surmounted before miRNA-based therapies become a reality is the issue of site-specific, safe, and effective delivery. The two main approaches at present for delivering miRNA therapies to target tissues, direct delivery of miRNA mimics or antigomirs and viral vector-encoded miRNA delivery, have specific limitations. The direct delivery approach, made possible by conjugating the oligonucleotide to cholesterol or coating it with liposomes or polycationic agents, avoids the immunogenic safety issues intrinsic to viral vector delivery. But it is challenged by the need for repeated dose delivery to achieve therapeutic effect. This becomes a critical issue if the route of delivery necessitates an invasive procedure. The viral vector-encoded miRNA delivery approach has the potential to simplify the delivery of multiple miRNA mimics/antagomirs in a single dose, due to the small size of the miRNA coding sequence, but is limited by its potential to trigger a host immune response, transient expression, and poor integration into the host genome (103,104). Future work must focus on developing more efficient delivery systems which minimize the number of healthy cells exposed to these therapies, promote good uptake/integration into the target tissue, and reduce the potential for off-target effects.

CONCLUSION

MiRNAs and their role in disease processes is a rapidly evolving field, as evidenced by the increasing body of literature. Their intricate involvement in the pathogenesis of many common diseases, including cancers, makes them ideal candidates for novel therapeutic strategies. In this review, we have highlighted the ways in which miRNAs can be used as disease modulators and drug targets. We have detailed the evidence to date that specific miRNAs can be associated with and manipulated for the treatment of liver disorders, diseases of the lung, breast cancer, and hematological malignancies. Given the increasing global interest in miRNAs, coupled with advances in molecular biochemistry and pharma technologies, miRNA-based therapeutic strategies look set to become the next generation of individualised targeted therapy adopted by the pharmaceutical and medical fields.

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