miRNA-Based Therapeutic Strategies

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Abstract Micro-RNAs (miRNAs) are short, non-coding RNA species, thought to act primarily through downregulation of target mRNA species with subsequent decrease in encoded proteins. Recent studies revealed that miRNAs play pivotal roles in physiology and disease, and therapeutic targeting has started being investigated. Generally, the upregulation of miRNAs is achieved through administration of synthetic miRNAs or administration of miRNA expressing vectors. The downregulation of miRNAs is achieved through administration of anti-sense nucleotides, often chemically modified to ensure stability and specificity. There are multiple potential limitations associated with the development and testing of miRNA-based therapeutics. These issues include, but are not limited to, off-target effect, avoidance from internal nucleases, and toxicity for miRNA therapy. In this review, we will discuss recent advances in miRNA based therapeutic strategies.

Keywords MicroRNA · Therapy · Antagomir · OncomiRNAs · Cancer

Introduction

MicroRNAs (miRNAs) are short (~22 nucleotides), single stranded, non-coding RNA species that were first reported to

have a function in 1993 [1]. It is believed that miRNAs exert their function mainly through binding of 6–8 nucleotide sequences (miRNA seed) to complementary sequences in target messenger RNA (mRNA), resulting in transcript degradation or translation inhibition, with the net effect of decreasing target protein amount [2•]. While some miRNA species are transcribed independently, others originate in introns or exons of genes and are transcribed in concert with mRNA species [3]. The transcribed RNA (pri-miRNA) contains the mature miRNA sequence (guide strand) as well as the complementary sequence (passenger strand). Within the nucleus, the enzyme Drosha cuts the pri-miRNA to pre-miRNA [4], which is then transferred out of the nucleus by a carrier protein, Exportin-5 [5]. Within the cytoplasm, pre-miRNA is processed by the enzyme Dicer to 20–25 base mature miRNAs [6]. Mature miRNA is then incorporated into the RNA-induced silencing complex (RISC), and interacts with target messenger RNA species [7]. Following initial reports of miRNA function, a plethora of studies identified salient roles for miRNAs in regulating a variety of physiologic and pathologic states. As the mechanism and role of miRNAs in human disease is gradually unraveled, recent studies also started exploring the role of miRNAs as therapeutics.

miRNA-based Therapeutic Strategies: Design, Pitfalls and Challenges

The process of developing miRNA-based therapeutics is somewhat similar to conventional drug discovery and testing protocols. An overview of miRNA-based therapeutic strategies is shown in Fig. 1. From a historical perspective, the development of conventional drugs generally starts with large screening tests, without preexisting hypotheses

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Fig. 1 Typical algorithm for identification and testing of miRNA species as therapeutics. The typical first step is identification of miRNA species that are differentially expressed between the diseased tissue of interest and normal tissue. Next, the miRNA is tested for function, as the screening alone cannot rule out that an miRNA is differentially expressed, but plays no etiologic role. Next, the miRNA species is optimized in terms of in vivo delivery, pharmacokinetics (PK), pharmacodynamics (PD), and absorption, distribution, metabolism, and excretion (ADME). Animal studies are then performed, followed by clinical trials

regarding mechanism of action. In contrast, most efforts to date to select miRNAs as targets for therapy were based on prior knowledge regarding disease specific miRNA dysregulation [8]. Although hypothesis-free screening of miRNAs as therapeutics can hypothetically be employed, the strategies used so far are likely based on the fact that miRNAs are intrinsic regulatory molecules, with defined, physiological functions. For example, miRNA-34 was found to have tumor suppressive roles in a variety of human tumors, and later became the focus of miRNA-based therapeutics [9, 10]. Following identification of miRNA species dysregulated in cancer, the next step usually entails functional characterization of the candidate miRNA species in vitro and in vivo through gain or loss of function studies. Gain of function can be accomplished through introduction of synthetic miRNA (either transfection or viral transduction). Loss of function can be accomplished by employing complementary antisense oligonucleotides, usually modified chemically to ensure stability and specificity.

miRNA Mimic-based Therapeutics: Technology and Challenges

RNA molecules are anionic, hydrophilic and are generally not taken up by cells via passive effusion [11]. In addition,

miRNAs may also be unstable in vivo due to rapid degradation by endogenous nucleases as well as rapid elimination through hepatic and renal metabolism and excretion [12]. One method to circumvent some of these limitations is local, rather than systemic, administration of RNA-based therapeutics [13]. Systemic administration is more challenging and efforts are underway to alter miRNA sequences through chemical modifications [12]. Atelocollagen coupling to miRNA molecules is such a modification, and offers several advantages: increased cellular uptake and nuclease resistance, without evoking interferon-based immune responses [14, 15]. miRNA can also be delivered as lipid-based nanoparticles [16, 17]. Lipid nanoparticles can reduce the negative electrical charge of RNA nucleotides and promote their cellular uptake [18]. Another strategy for efficient systemic delivery of miRNA therapeutics is through viral vectors [19]. Adeno-associated virus vectors [20] or lentiviral vectors [21] can be used to transfer miRNAs to cells. Specificity of viral-mediated delivery continues to be a challenge, and current approaches employ viral serotypes demonstrated to more selectively transduce the cell type of interest, as well as using specific promoters [22, 23].

miRNA Inhibitor-based Therapeutics: Technology and Challenges

To reduce endogenous miRNA levels, anti-sense oligonucleotides [24], called AMO (anti-miRNA antisense oligomer), also called antagomir [25], are usually employed. Various methods have been tested to render AMO constructs more stable in vivo and to ensure adequate tissue availability and specificity. For example, 2'-O-methyl (2'-OMe) modified RNA is chemically more stable than unmodified RNA, and more resistant to nucleases due to the methylated hydroxyl base [26–28]. Also, 2'-O-methoxyethyl (2'-MOE) phosphorothioate and 2'-fluoro/2'-methoxyethyl (2'-F/MOE) modifications have been employed to increase the efficiency of the synthetic oligonucleotide against the target miRNA [29–32]. In addition, conjugation with cholesterol appears to improve activity of the nucleotide sequence [27, 33, 34]. Locked nucleic acid (LNA) is another method to modify antisense oligonucleotides, where the 2' oxygen and 4' carbon of the nucleotide are bridged with methylene and form a cyclic structure. Because LNA is locked by this bridge, it does not transition between conformations, in contrast to the naked nucleotide sequence. LNA is more resistant to the endogenous nucleases, has stronger affinity to the target nucleotide [35, 36], and displays lower toxicity [37]. Last, peptide nucleic acid (PNA) is an artificially synthesized polymer that has a peptide structure in the main chain and similarity to DNA and RNA [38]. Because it does not have an organophosphate moiety, these constructs bind to target nucleotide sequences more tightly than nucleotide/

nucleotide binding. In addition, PNA appears to be relatively stable and can be administered systemically with relatively low toxicity [39].

miRNA Sponge and miRNA Masking: An Indirect Method to Reduce Endogenous miRNA Species

miRNA sponges and masking are relatively new strategies employed to downregulate miRNAs. MiRNA sponges contain multiple target sites complementary to a target miRNA that is deemed to be of potential therapeutic relevance [40, 41]. miRNA masking entails the design and administration of sequences complementary to a miRNA binding site in the 3' UTR of the target mRNA. Through the competitive inhibition of the interaction between the miRNA and mRNA species, the miRNA mask reduces the activity of endogenous miRNA [42].

miRNA Off-target Effects

Decades of conventional drug discovery and design suggest that drug off-target effects are a salient aspect of the development process. Off-target effect challenges are applicable, to a certain extent, to developing miRNA-based therapeutics [43]. Small RNA species, of the miRNA as well as siRNA type, can bind not only to fully complementary nucleotide sequences, but also to similar sequences [44]. Off-target effects may cause unexpected consequences, sometimes completely negating the utility of a particular small RNA species as therapeutics. The probability of off-target effects can be predicted for small interfering (si) RNA [45, 46], however, the true magnitude of off-target effects cannot be fully known until in vivo studies are performed. Off-target studies with miRNAs are, in comparison with siRNA studies, not as well developed. In addition, although not proven on a large scale, we believe that miRNA species may escape the “off-target effects curse”, at least to a certain extent. The main advantage of miRNA species when compared to synthetic siRNA species is the fact that miRNAs are intrinsic molecules. If we hypothesize that their modus operandi is through effects of small amplitude exerted on multiple targets simultaneously, then, by definition, their effects cannot be considered off-target. While in vitro and some preliminary in vivo studies suggest that this hypothesis is true, further in vivo studies are clearly needed [47].

Specific miRNA-based Therapeutic Advances

Cancer

Dysregulated miRNA species have been reported to be involved in carcinogenesis, cancer progression, and drug

Table 1 Representative oncomiRNA and TS miRNA species

Cancer	OncomiRNA	TSmiRNA
Breast cancer	mir-21 [9]	mir-34b [78]
Colorectal cancer	mir-211 [79]	mir-33a [80]
Gastric cancer	mir-192 [81], mir-215 [81]	mir-125a [82]
Glioma	mir-21 [83]	mir-34a [10]
Hepatocellular carcinoma	mir-21 [50]	mir-26a [22•]
Leukemia	mir-100 [84]	mir-33a [80]
Lung cancer	mir-31 [85]	mir-101 [86]
Ovarian cancer	mir-21 [87]	let-7 [88]
Pancreatic cancer	mir-21 [89]	mir-150 [90]
Prostate cancer	mir-32 [91]	mir-34a [92]

resistance [48, 49]. Theoretically, miRNA species upregulated in cancer (oncomiRNAs) as well as downregulated in cancer (tumor suppressor miRNAs, or TSmiRNAs) can be targeted for miRNA based therapeutics. Table 1 contains a list of the more studied oncomiRNAs as well as TSmiRNAs.

Therapeutic miRNA-21 Blockade in Cancer

miRNA-21, located in a coding gene, transmembrane protein 49, is one of the most studied miRNA species in human cancer. The collective knowledge from several studies indicates that miRNA-21 regulates a multitude of target genes, most of which are known to exert tumor suppressor effects. The targets of miRNA-21 include phosphatase and tensin homolog (PTEN), sprouty homolog 1 (SPRY1), B cell CLL/lymphoma 2 (BCL2), and others [50]. miRNA-21 was associated with a variety of cancers, including leukemia [51], lung [52], liver [50], stomach [53], pancreas [54], and brain [55]. Several in vitro as well as in vivo studies demonstrated that reducing miRNA-21 levels with anti-sense nucleotides results in delayed cancer growth in gastric cancer [53], gliomas [56] and other cancers.

Therapeutic miRNA-26 Upregulation in Hepatocellular Carcinoma

miRNA-26 was first implicated in liver cancers in a profiling study performed on a large cohort of human specimens [57]. Additional mRNA profiling also identified a possible involvement of miRNA-26 in nuclear factor Kappa B and Interleukin-6 pathways, further suggesting an etiologic role for this miRNA in liver cancer genesis or homeostasis. Building on prior data regarding the downregulation of miRNA-26a in liver cancers, another group investigated the

usage of miRNA-26a as liver cancer therapeutics in vivo [22•]. This is the first study to successfully use adeno-associated viruses (AAVs) to deliver miRNA in vivo to a mouse model of hepatocellular carcinoma, and resurrected the interest in AAVs as delivery methods for miRNAs. This study, however, also raises an interesting point regarding organ and tissue specific expression of miRNAs. Specifically, miRNA-26 does not appear to be underexpressed in all human tumors, suggesting that miRNA expression is highly tissue-dependent. This finding advocates caution in using miRNAs for therapeutics, in particular if the specificity of tissue delivery is not finely tuned. For example, miRNA-26 was found to be up-regulated in glioblastoma and overexpression of miRNA-26 increases tumor growth and proliferation [58]. Although it is currently not known if upregulation of miRNA-26 in normal cells can, under certain conditions, promote de novo cancer genesis, these findings are of unquestionable concern. It is conceivable that liver-specific delivery of miRNA-26 may be possible, however, hepatocellular cancer cell, or hepatocyte-specific delivery may be significantly more difficult to accomplish. The need for hepatocyte-specific delivery was further suggested by a recent study, that found overexpression of miRNA-26a in human cholangiocarcinoma (CCA) [59]. These findings are more so concerning since the same study reported an etiologic role for miRNA-26 in CCA growth. These recent data raise a serious concern regarding miRNA-26 supplementation in hepatocellular cancers and further emphasize the need for tissue and cell type specific targeting of miRNA based therapies.

Therapeutic Let-7 Upregulation in Cancers

The Let-7 family is one of the first identified miRNAs, and it is highly conserved in a wide range of species, suggestive of its functional role [60]. Further studies identified a tumor suppressive role for this miRNA family in a variety of cancers [61–63]. Mechanistic studies found that Let-7 directly binds to the 3'UTR of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) resulting in its downregulation [64]. In addition, let-7 has been reported to downregulate other oncogenes, such as high-mobility group AT-hook 2 (HMGA2) and v-myc myelocytomatosis viral oncogene homolog (MYC) [64]. Gain of function studies demonstrated that induction of let-7 inhibits DNA replication and regulates apoptosis [64]. These data suggested that let-7 is an attractive miRNA for treatment for cancer. In vivo upregulation of let-7 in various cancers, such as prostate cancer [65], and lung cancer by nasal administration [66], by inducible vector [67], or exogenous delivery [23], induced decreased cell proliferation. Further studies are needed to accurately assess the utility of let-7 based miRNA therapeutics in vivo.

Hepatitis C infection

Therapeutic miRNA-122 Blockade in Hepatitis C

miRNA-122 is one of the most studied miRNAs as in vivo therapeutics. Several studies demonstrated that miRNA-122 plays a pivotal role in a variety of liver-centric physiologic and pathologic processes. In vivo antisense blockade of miRNA-122 in liver results in significantly decreased plasma cholesterol levels in mice, as well as in non-human primates [29, 35]. Interestingly, these studies also demonstrated that miRNA blockade in vivo can have an enduring effect [35]. Additional studies revealed yet another surprising role for miRNA-122 in Hepatitis C (HCV) infection. In vitro studies demonstrated that miRNA-122 expression is a requirement for HCV replication through direct binding to sites within the 5'UTR of HCV [68]. Following these results, *Santaris Pharma* introduced a locked nucleic acid (LNA) miRNA-122 antisense sequence and preliminary experiments in non-human primates demonstrated a significant reduction of HCV titer in the higher dose group [69•]. Also of note, the effect was maintained over several weeks while therapy was administered. In addition, there was no evidence of liver toxicity or HCV mutations. These preliminary results formed the basis of a recently concluded phase 2a study led by *Santaris Pharma*, published in abstract form at the 2011 Annual meeting of the American Association for the Study of Liver Diseases (AASLD). The authors reported that 4 of the 9 patients on the high dose of anti-miR-122 therapy had no detectable HCV titer 4 weeks after initiation of therapy.

These studies also shed some light onto other aspects of miRNA functioning. Interestingly, miR-122 binds to the 5'UTR of HCV, not to the typical 3'UTR location. In addition, miR-122 binding appears to stimulate, rather than inhibit the expression of HCV [68]. Another interesting observation stemmed from measuring and correlating miRNA-122 levels in serum of HCV patients with serum HCV titers [70]. Interestingly, there was no correlation between serum levels of HCV and miRNA-122. In other words, if the first step in identifying a miRNA that may inhibit HCV were a profiling study, miRNA-122 would not have been found. This study raises an important question: is the current protocol involving profiling human tissues to identify dysregulated miRNA species with the purpose of finding candidate miRNAs for therapeutics the best approach? Would a typical drug screening approach, where knowledge regarding miRNA levels in diseased tissue compared to normal tissue is irrelevant, work better?

A significant area of concern with further developing miRNA-122 blockade for human use stems from the observation that miRNA-122 has low levels of expression

in HCC [71]. Down-regulation of miRNA-122 is observed in patients with HCC and over expression of miRNA-122 inhibits tumor proliferation in HCC cell lines [72]. Moreover, two recent studies demonstrated that miR-122 deletion in mice results in hepatosteatosis, fibrosis and HCC development [73, 74]. In our view, these seemingly contradictory results do not necessarily suggest the presence of off target effects, but they rather underline the paramount importance of tissue and temporal specific miRNA manipulation. In our opinion, these studies argue that miRNA-122 levels may play roles of vastly different magnitude on a variety of processes (growth, stemness, fibrosis, cancer development, Hepatitis C infection) dependent on cell type as well as on developmental stage.

Fibrosis

Therapeutic miRNA-21 Blockade in Heart Fibrosis and miRNA-21 Upregulation in Abdominal Aortic Aneurysms (AAA)

In response to injury, fibroblasts produce extracellular matrix as a normal, physiologic response. However, uncontrolled matrix production leads to fibrosis and to a variety of fibrosis-related complications, such as idiopathic pulmonary fibrosis, liver cirrhosis, scleroderma, bowel strictures or bone marrow failure. Transforming growth factor beta (TGF- β) is thought to be a central mediator of fibrosis, and miRNA-21 is a known modulator of TGF- β pathway. Several groups, therefore, studied the utility of miRNA-21 blockade in various fibrosis models. In a mouse model of lung fibrosis, reminiscent of human idiopathic pulmonary fibrosis, antisense blockade of miRNA-21 was shown to diminish the magnitude of the fibrotic process [75]. In a different study, miRNA-21 was found to be upregulated in activated fibroblasts of the failing heart, with a direct impact onto the extent of heart fibrosis [76•]. Based on these findings, an in vivo study of a mouse model of pressure-overload-induced heart disease found that administration of a miRNA-21 antisense construct reduces the extent of heart fibrosis and overall heart function [76•]. In sharp contrast, a different study in a mouse model of abdominal aortic aneurysm (AAA) found that miRNA-21 blockade increased the size of the AAA and conversely, miRNA-21 overexpression limited the expansion of AAA [21]. Although these findings are encouraging for the potential utility of miRNA-21 overexpression for patients with AAA, clear concerns exist regarding potential side effects from upregulating miRNA-21 in liver (where high levels are associated with HCC) or heart (where high levels are associated with increased fibrosis).

Lipid Metabolism

Therapeutic miRNA-33 Blockade

miRNA-33a and miRNA-33b are located within the intron of sterol regulatory element-binding proteins (SREBP) 2 and SREBP1, respectively. SREBPs are key genes for cholesterol synthesis and uptake. Interestingly miRNA-33a and miRNA-33b were also found to play a pivotal role in lipid metabolism, through interaction with adenosine triphosphate-binding cassette transporter A1 (ABCA1) and ABCG1, regulators of high density lipoprotein synthesis [77•]. Therefore, systemic antagonism of miRNA-33 is expected to improve lipid metabolism and reduce plaque formation in vessels. Mice on a western diet injected with LNA-antisense to miRNA-33 demonstrated an increased in plasma levels of high density lipoprotein (HDL) [77•]. Moreover, in vivo inhibition of miRNA-33 was found to have a reduction in atherosclerotic plaques size and lipid content, through raising ABCA1 levels in the liver, as well as in the plaque macrophages [32].

Spinal and Bulbar Muscular Atrophy (SBMA)

Therapeutic miRNA-196a Upregulation in SBMA

SBMA, also known as Kennedy–Alter–Sung disease, is an inherited neurodegenerative disorder that results in proximal muscle weakness, atrophy, contractions and bulbar involvement. So far, there are no curative treatments for SBMA. A recent study evaluated AAV-mediated delivery of miRNA-196a in a mouse model of SBMA [20]. CUGBP, Elav-like family member2 (CELF2) is known to bind to the mutated androgen receptor and stabilize it. miRNA-196a was found to be overexpressed in spinal cords of SBMA mice compared to controls, perhaps as a protective, albeit insufficient, mechanism. In addition, miRNA-196 binds to CELF2 and represses it, resulting in a lower amount of mutated androgen receptor, with amelioration of SBMA. An AAV vector expressing miRNA-196a delivered in vivo to SBMA mice induced clinical improvement and represents an exciting new therapeutic venue to be explored in SBMA clinical trials.

Conclusion

miRNA-based therapeutics is an emerging field showing significant promise. Studies in mice, non-human primates and early trials in humans clearly demonstrate that there is potential for developing miRNAs into valuable therapeutics. There are, however, multiple areas of concern that deserve further investigation. For example, it is not entirely

clear if miRNAs should be viewed similarly to siRNAs or chemical compounds in terms of off-target effects. miRNAs appear to be built-in regulatory molecules, designed to exert global modulation through moderate effects on a multitude of targets. Therefore, development of miRNAs into therapeutics may be less prone to true off-target effects. Nonetheless, while true off target effects could be rare, the same cannot be stated regarding side effects. There are several examples (miRNA-21, miRNA-122, and others) where downregulation in a diseased tissue appears beneficial, but data from a different tissue or disease indicate severe potential side effects of decreasing same miRNA levels, probably in particular if chronic treatment is necessary. Other areas of further research include efficient in vivo delivery. In vivo miRNA upregulation has been attempted by employing viral vectors, however, care must be exercised, similar to other viral-based in vivo gene therapy efforts. In addition, while some specificity can be attained based on the AAV serotype, further efforts are necessary to increase delivery specificity to the organ of interest alone, or, even to the cell of interest. However, given the relatively short period of time since discovery of miRNAs, the progress appears sufficient to justify optimism regarding developing novel therapeutics based on miRNAs.

Conflicts of interest No potential conflicts of interest relevant to this article were reported.

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