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REVIEW

siRNA Versus miRNA as Therapeutics for Gene Silencing

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Discovered a little over two decades ago, small interfering RNAs (siRNAs) and microRNAs (miRNAs) are noncoding RNAs with important roles in gene regulation. They have recently been investigated as novel classes of therapeutic agents for the treatment of a wide range of disorders including cancers and infections. Clinical trials of siRNA- and miRNA-based drugs have already been initiated. siRNAs and miRNAs share many similarities, both are short duplex RNA molecules that exert gene silencing effects at the post-transcriptional level by targeting messenger RNA (mRNA), yet their mechanisms of action and clinical applications are distinct. The major difference between siRNAs and miRNAs is that the former are highly specific with only one mRNA target, whereas the latter have multiple targets. The therapeutic approaches of siRNAs and miRNAs are therefore very different. Hence, this review provides a comparison between therapeutic siRNAs and miRNAs in terms of their mechanisms of action, physicochemical properties, delivery, and clinical applications. Moreover, the challenges in developing both classes of RNA as therapeutics are also discussed.

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The term "non-coding RNA" is commonly employed for RNA that does not encode a protein.[1] Although the current understanding of these RNA molecules represents perhaps only the tip of the iceberg, with the rapid development of molecular biotechnology, noncoding RNAs are increasingly found to have far more important functions than previously recognized and many new classes of noncoding RNA have been identified. Among them, small interfering RNAs (siRNAs) and microRNAs (miRNAs) have attracted considerable attention because their role in gene regulation makes them likely targets for drug discovery and development. Indeed, the therapeutic potential of siRNAs and miRNAs has been demonstrated in the treatment of many different diseases including cancers[2–4] and infections.[5–7] Compared with conventional small therapeutic molecules, siRNAs and miRNAs offer the advantages of being highly potent and able to act on "non-druggable" targets (for example, proteins which lack an enzymatic function or have a conformation that is inaccessible to traditional drug molecules),[8] as they can be designed to affect virtually any gene of interest.

Therapeutic approaches based on siRNA involve the introduction of a synthetic siRNA into the target cells to elicit RNA interference (RNAi), thereby inhibiting the expression of a specific messenger RNA (mRNA) to produce a gene silencing effect.[9] By contrast, miRNA-based therapeutics comprise two approaches: miRNA inhibition and miRNA replacement. The former approach resembles antisense therapy,[10] with synthetic single stranded RNAs acting as miRNA antagonists (also known as antagomirs or anti-miRs) to inhibit the action of the endogenous miRNAs. In the replacement approach, synthetic miRNAs (also known as miRNA mimics) are used to mimic the function of the endogenous miRNAs.[11] It thus leads to mRNA degradation/inhibition, and produces a gene silencing effect. This review focuses on the therapeutic approach achieved by gene silencing, and so only the miRNA replacement approach is discussed and compared with siRNA. The therapeutic potentials and applications of the miRNA inhibition approach have been reviewed previously.[12–14]

siRNAs and miRNAs have similar physicochemical properties but distinct functions (**Table 1**). Both are short RNA duplexes that target mRNA(s) to produce a gene silencing effect, yet their mechanisms of action are distinct. As a result, the requirements for sequence design and therapeutic applications of siRNAs and miRNAs are different. On the other hand, for clinical development, the two types of small RNA molecules face a similar set of barriers: poor stability *in vivo*, delivery challenges and off-target effects[15]; and so, the same strategies can be employed to improve their *in vivo* efficacy.

Gene silencing mechanism of siRNA and miRNA

RNA interference and siRNA

RNAi is a natural cellular process that silences gene expression by promoting the degradation of mRNA. It plays an important role in gene regulation and innate defense against invading viruses.[16] RNAi was first described by Fire and Mello based on their Nobel prize winning study investigating the mechanisms for effective gene inhibition by exogenous RNA in *C. elegans*.[17] According to their observations, long double-stranded RNA (dsRNA) mediates potent and specific silencing of homologous genes. It appeared later that a similar process also occurs in mammals.[18] After years of investigation, the mechanism underlying RNAi is better understood (**Figure 1**). In general, the dsRNA (either transcribed from cellular genes or infecting pathogens, or artificially introduced into the cells) is processed by a specialized ribonuclease

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Table 1 Comparison of general properties between siRNA and miRNA

	siRNA	miRNA
Prior to Dicer processing	Double-stranded RNA that contains 30 to over 100 nucleotides	Precursor miRNA (pre-miRNA) that contains 70–100 nucleotides with interspersed mismatches and hairpin structure
Structure	21–23 nucleotide RNA duplex with 2 nucleotides 3'overhang	19–25 nucleotide RNA duplex with 2 nucleotides 3'overhang
Complementary	Fully complementary to mRNA	Partially complementary to mRNA, typically targeting the 3' untranslated region of mRNA
mRNA target	One	Multiple (could be over 100 at the same time)
Mechanism of gene regulation	Endonucleolytic cleavage of mRNA	Translational repression
		Degradation of mRNA
		Endonucleolytic cleavage of mRNA (rare, only when there is a high level of complementary between miRNA and mRNA)
Clinical applications	Therapeutic agent	Drug target
		Therapeutic agent
		Diagnostic and biomarker tool

(RNase) III-like enzyme named Dicer in the cytoplasm into a smaller dsRNA molecule. This short dsRNA molecule is known as the siRNA, which has 21–23 nucleotides with 3′ two-nucleotide overhangs. The siRNA interacts with and activates the RNA-induced silencing complex (RISC). The endonuclease argonaute 2 (AGO2) component of the RISC cleaves the passenger strand (sense strand) of the siRNA while the guide strand (antisense strand) remains associated with the RISC. Subsequently, the guide strand guides the active RISC to its target mRNA for cleavage by AGO2. As the guide strand only binds to mRNA that is fully complementary to it, siRNA causes specific gene silencing.[16,19]

Since the discovery of RNAi, dsRNAs have been used as research tools to study the gene functions of different cell types. However, in mammalian cells, the delivery of exogenous, long dsRNAs (over 30 nucleotides) is associated with the activation of the interferon (IFN) pathway,[20] which is part of the defense mechanism against viral infection. The long dsRNAs bind and activate protein kinase R (PKR), which in turn stimulate a plethora of genes belonging to the IFN pathway, resulting in nonspecific mRNA degradation and apoptosis.[21] An *in vitro* study in mammalian cells, including human cell cultures, showed that the direct introduction of synthetic siRNAs, instead of the long dsRNAs (thus skipping the step of Dicer processing), leads to effective RNAi without the complication of activating the IFN response.[18] In view of this finding, siRNAs have become useful tools to inactivate target gene expression. However, later studies suggest that synthetic siRNAs may also induce partial IFN response and innate immune responses.[22,23] As this effect can be either sequence-dependent or -independent, special care must be taken when designing siRNA therapeutics. Alternatively, short hairpin RNAs (shRNAs) can be used to achieve a specific gene silencing effect via the RNAi mechanism.[24] shRNAs are stem-loop RNAs, which are expressed in the nucleus, typically through the delivery of viral vectors. Once expressed, they are transported to the cytoplasm for further processing, and subsequently loaded into the RISC for specific gene silencing activity in the same manner as synthetic siRNAs. However, the requirement of viral vectors for shRNA expression poses safety concerns in therapeutic applications, which are discussed in section "Viral vectors".

Gene silencing mediated by miRNA

Similarly to siRNAs, miRNAs also inhibit gene expression in a post-transcriptional manner. Although the gene silencing effects of siRNAs and miRNAs are distinct, the distinction has been obscured because they are associated with common enzymes (*e.g.*, Dicer and RISC) and their functions overlap with each other to a certain extent. The major difference between siRNAs and miRNAs is that the former inhibit the expression of one specific target mRNA while the latter regulate the expression of multiple mRNAs. A considerable body of literature now classifies miRNAs as RNAi molecules.[15,25–27]

The first miRNA was discovered in 1993 in a study examining developmental regulatory genes in *C. elegans*.[28] Soon after its discovery, miRNA was quickly found to be a class of small RNA molecules that negatively regulate gene expression (**Figure 1**). miRNA gene transcription is carried out by RNA polymerase II in the nucleus to give primary miRNA (pri-miRNA), which is 5′ capped, 3′ polyadenylated RNA with double-stranded stem-loop structure. The pri-miRNA is then cleaved by a microprocessor complex (comprising Drosha and microprocessor complex subunit DCGR8) to form precursor miRNA (pre-miRNA), which is a duplex that contains 70–100 nucleotides with interspersed mismatches and adopts a loop structure. The pre-miRNA is subsequently transported by Exportin 5 from the nucleus to the cytoplasm, where it is further processed by Dicer into a miRNA duplex of 18–25 nucleotides. The miRNA duplex then associates with the RISC forming a complex called miRISC. The miRNA duplex is unwound, releasing and discarding the passenger strand (sense strand)—unlike in the processing of siRNA, in which the AGO2 of the RISC causes the cleavage of the passenger strand of siRNA. The mature single-stranded miRNA guides the miRISC to the target mRNAs. The miRNA binds to the target mRNAs through partial complementary base pairing with the consequence that the target gene silencing occurs via translational repression, degradation, and/or cleavage.[25,29]

Recognition of mRNA targets by siRNA and miRNA

To elicit RNAi, the siRNA must be fully complementary to its target mRNA (**Figure 2**). The complementary binding activates the AGO2, which then cleaves the phosphodiester

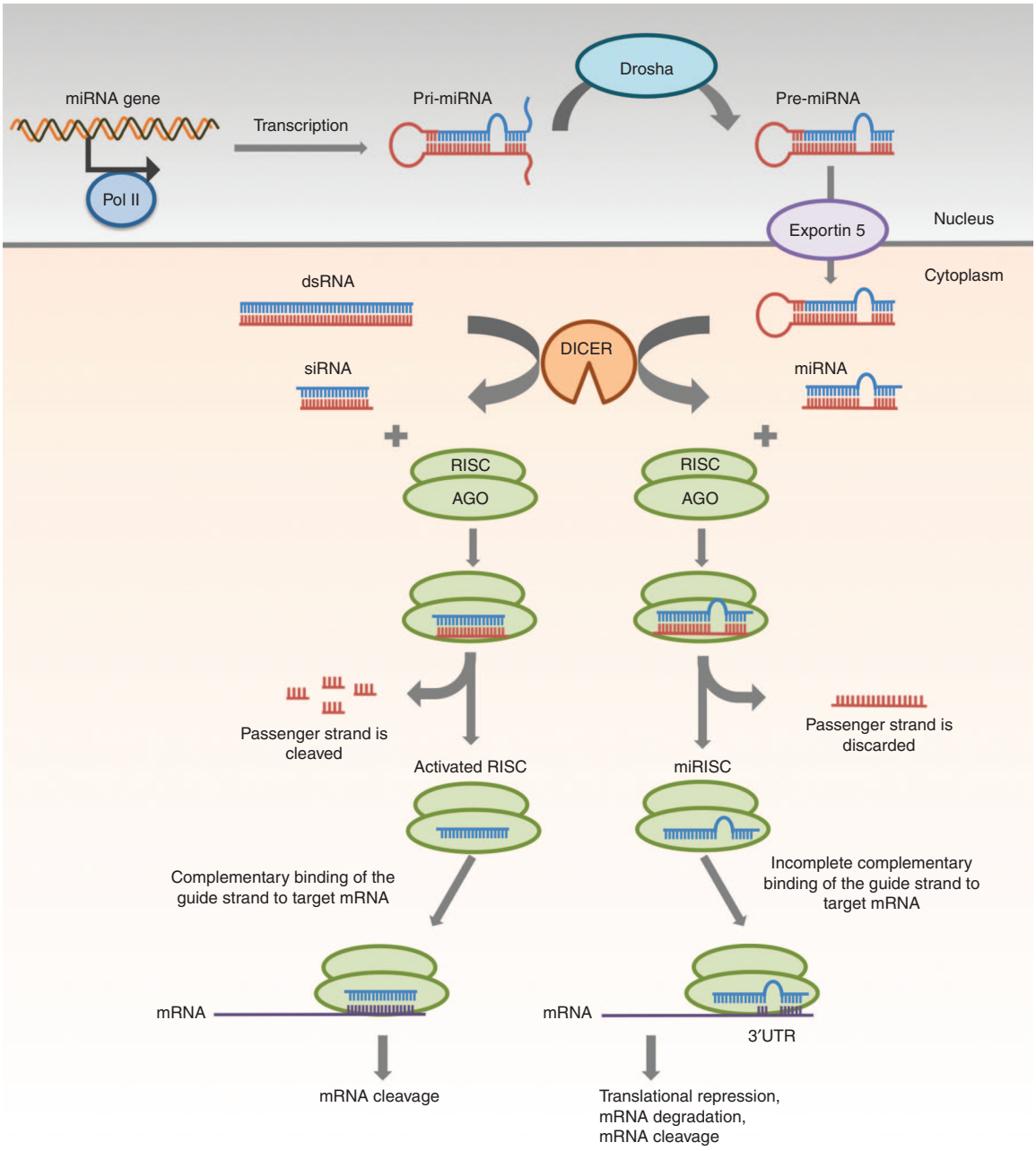

Figure 1 Gene silencing mechanisms of siRNA and miRNA. siRNA: dsRNA (either transcribed or artificially introduced) is processed by Dicer into siRNA which is loaded into the RISC. AGO2, which is a component of RISC, cleaves the passenger strand of siRNA. The guide strand then guides the active RISC to the target mRNA. The full complementary binding between the guide strand of siRNA and the target mRNA leads to the cleavage of mRNA. **miRNA:** Transcription of miRNA gene is carried out by RNA polymerase II in the nucleus to give pri-miRNA, which is then cleaved by Drosha to form pre-miRNA. The pre-miRNA is transported by Exportin 5 to the cytoplasm where it is processed by Dicer into miRNA. The miRNA is loaded into the RISC where the passenger strand is discarded, and the miRISC is guided by the remaining guide strand to the target mRNA through partially complementary binding. The target mRNA is inhibited via translational repression, degradation or cleavage.

backbone of the mRNA between bases 10 and 11 relative to the 5′end of the guide strand.[30] The mRNA fragments generated are subsequently degraded by different exonucleases.[31] By contrast, the target recognition of miRNA is more complex, as different binding sites and different degree of complementarity between the miRNA and the target RNA exist. This is a consequence of imperfect base pairing; miRNA only needs to be partially complementary to its target mRNA. The complementary pairing between mRNA and the mature miRNA typically occurs at the 3′ untranslated region (UTR) of the former and the seed region (nucleotides 2–7 from the 5′ end) of the latter (**Figure 2**).[32,33] Other miRNA binding sites, such as the centered sites, 3′ supplementary sites and bulged sites, are considered to be atypical.[32,34,35] Since miRNA-mRNA

recognition does not require perfect pairing, one miRNA strand can recognize an array of mRNAs, and hence miRNA has the characteristic of having multiple targets. For example, a microarray analysis showed that miRNA-124, which is preferentially expressed in brain tissues, can downregulate 174 annotated genes.[33] Due to the partially complementary base pairing between mRNA and miRNA, AGO2 of the miRISC is not activated. Instead, the silencing of the mRNA targets of miRNA occurs through translation repression, or degradation by deadenylation, decapping or exonuclease action.[36] In rare cases, high level of complementary between mRNA and miRNA leads to the endonucleolytic cleavage of mRNA by AGO protein, a mechanism that is similar to siRNA-mediated gene silencing.[37]

siRNA and miRNA as therapeutic agents

The specific gene silencing effect of siRNAs makes them useful tools for target identification and validation in drug discovery and development.[38,39] Since miRNAs have multiple mRNA targets and the disruption of their functions contributes to the development of many diseases including cancers, neurodegenerative disorders and cardiovascular diseases, their clinical use as biomarkers and in diagnostics is rapidly developing.[40] Furthermore, both siRNAs and miRNAs have huge potential as therapeutic agents. They can overcome the major limitation of traditional small drug molecules, which can only target certain classes of proteins. Even for protein-based drugs including monoclonal antibodies that are highly specific, their targets are mainly limited to cell-surface receptors or circulating proteins. By contrast, siRNAs and miRNAs can downregulate the expression of virtually all genes and their mRNA transcripts. Since many diseases result from the expression of undesired or mutated genes, or from overexpression of certain normal genes, the discovery of siRNA and miRNA opens up a whole new therapeutic approach for the treatment of diseases by targeting genes that are involved causally in the pathological process. A comparison between conventional small molecules, protein-based therapeutic agents and siRNA/miRNA-based drugs is summarized in **Table 2**. Although the therapeutic potential of siRNAs and miRNAs is promising, different sets of hurdles retard their development into clinical use. Some of these challenges, such as problems regarding stability and poor efficiency of delivery, are similar for both RNA molecules.

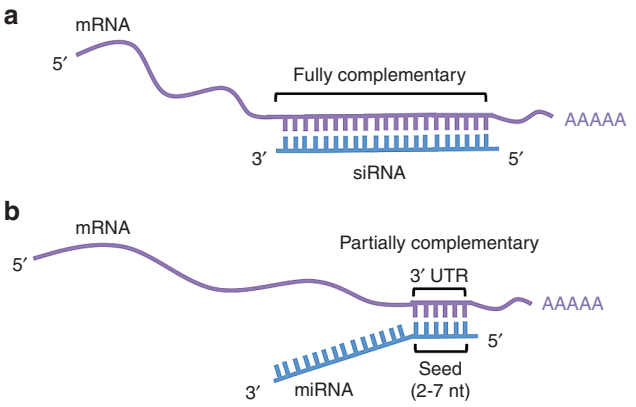

Figure 2 Target recognition by siRNA and miRNA. (**a**) siRNA is usually fully complementary to the coding region of its target mRNA; (**b**) miRNA is partially complementary to its target miRNA. Complementary binding usually occurs at the seed region (nucleotides (nt) 2–7 of the 5' end) of miRNA and the 3' UTR of the target mRNA.

Design of therapeutic siRNA

The first essential step for successful siRNA therapy is the design of a siRNA sequence that is potent and specific to the intended mRNA to minimize any off-target effect. A conventional siRNA consists of 19–21 nucleotides with two nucleotide overhangs at the 3′ end, usually TT and UU, which are important for recognition by the RNAi machinery.[41] Increasing the length of the dsRNA may enhance its potency, as demonstrated by an *in vitro* study that dsRNAs with 27 nucleotides were up to 100 times more potent than the conventional siRNAs with 21 nucleotides.[42] The long dsRNAs require processing by Dicer into the shorter siRNAs (hence they are termed as "Dicer-ready" or "Dicer-substrate" siRNAs), which are more efficiently loaded into the RISC, thus facilitating the subsequent gene silencing mechanism.[21,42–44] On the other hand, dsRNAs longer than 30 nucleotides can activate the IFN pathway[20] and should be avoided for therapeutic applications.

The gene silencing efficiency of the siRNA varies greatly, depending on the region of the mRNA to which they are complementary. An understanding of this relationship can permit the design of a siRNA sequence with optimal efficacy, and hence the rational design of effective siRNA sequences has been a focus of research. While many siRNA design algorithms have emerged in recent years to predict efficacy,[45,46] it is nevertheless essential to validate the gene silencing efficiency of siRNA experimentally. Some commonly used

Table 2 A comparison between small molecules, protein-based drugs (including monoclonal antibodies) and siRNA/miRNA-based drugs

Properties	Small molecules	Protein-based drugs	siRNA/miRNA-based drugs
Nature of action	Activation or inhibition of targets	Activation or inhibition of targets	Inhibition of targets
Site of target proteins	Extracellular and Intracellular	Mainly extracellular	Virtually any sites
Selectivity and potency	Variable (depending on binding-site and ligand specificity, their affinity and efficacy etc.)	Highly specific and potent	Highly specific and potent
Lead optimization	Slow	Slow	Rapid
Manufacture	Easy	Difficult	Easy
Stability	Stable	Unstable	Unstable
Delivery	Easy	Difficult	Difficult

Data taken from ref. 201.

Table 3 A summary of commonly employed strategies to enhance the efficacy and specificity of siRNAs, and to reduce the off-target effects

siRNA feature	Strategy	Description
Strand selection	Apply asymmetry rule	Strand with a relatively unstable 5' end is selected as guide strand
	Utilize 5′ nucleotide preference	Strand with U or A at position one at the 5' end is preferentially selected as guide strand
Activity	Manipulate G/C content	G/C content is ideally between 30–64%
		G/C stretches of >9 nucleotides should be avoided
Off-target	Reduce siRNA concentration	Lowest possible siRNA concentration to achieve a therapeutic effect is used
	Use multiple siRNAs	siRNAs with different sequences for targeting the same mRNA are pooled for therapeutic effect
miRNA-like effect	Avoid sequences similar to miRNA	Avoid seed sequences of miRNA that have already been identified
Immune stimulation	Avoid immune stimulatory motifs	Avoid U-rich sequences and motifs that contain:
		GUCCUUCAA
		UGUGU
		UGU
		UGGC

strategies for the design of therapeutic siRNAs are summarized in **Table 3**.

Strand selection. To ensure effective gene silencing, the siRNA must be correctly orientated and loaded into the AGO of the RISC in order for the passenger strand to be cleaved and discarded, so that the guide strand that is complementary to the target mRNA remains bound to the active RISC and directs it to the target mRNA. The guide strand of the RNA duplex is determined during the AGO loading step.[47] However, both strands in the RNA duplex could potentially be loaded into the AGO as the guide strand. An incorrect loading orientation results in the intended guide strand being discarded and off-target effects are produced as the remaining strand (the intended passenger strand) base-pairs to the nonintended mRNA. Since this phenomenon can occur with both siRNA and miRNA,[48–50] the RNA duplex needs to be carefully designed to warrant correct guide strand selection by the RISC. Two major sequence parameters are known to determine the guide strand selection: (i) the asymmetry rule and (ii) 5′ nucleotide preference; both of which can be applied to siRNA as well as miRNA design.

The *asymmetry rule* is based on the finding that the relative thermodynamic stability of the two ends of the duplex contributes to the selection of the strand to be loaded into AGO.[48,51] The strand with a relatively unstable 5′ end (*i.e.*, higher A/U content) is selected as guide strand while the strand with a more stable 5′ end is discarded as the passenger strand. For this reason, RNA duplexes should always be designed with the intended guide strand having the less stable 5′ end. In addition to the asymmetry rule, the *5′ nucleotide preference* is also important in correct AGO loading. AGO proteins appear to have a preference for the strand with a U, or less favorably, an A at position one at the 5′ end as the guide strand. Therefore, the guide strand should ideally contain a U or A at the 5′end, whereas the passenger strand should always contain C and G at the 5′ end to minimize the risk of being incorrectly selected as a guide strand.[52]

Efficiency affected by G/C content. The overall G/C content of the siRNA influences the siRNA activity,[53] although the importance of this influence is still debated. The G/C content affects the overall duplex thermodynamic stability as well as target site accessibility; siRNAs with very high G/C content appear to be less functional.[54] Some studies suggest that the optimal G/C content of siRNA is around 30–50%, while others show that siRNAs with G/C contents of about 60% are highly efficient.[55,56] As a general guideline, the G/C content of siRNA is ideally between 30 and 64%.[57] Furthermore, sequences with G/C stretches of nine or more nucleotides should be avoided as this may reduce the gene silencing efficiency of siRNA.[58]

Minimizing off-target effect. Although one of the distinctive features that differentiate siRNA from miRNA is that siRNA is designed to silence the expression of a specific target mRNA, siRNA may lead to the downregulation of unintended, unpredicted targets, resulting in off-target effects. Indeed, one of the major challenges of siRNA therapy is to reduce off-target effects, as these compromise the therapeutic effect, specificity and can even lead to cell death.[59]

The most common type of off-target effect of siRNA is the miRNA-like effect.[60–63] This occurs when the 5′ end of the guide strand of siRNA is complementary to the 3′UTR of the mRNA (reminiscent of the target recognition by the seed region of miRNA).[64] In some situations, this off-target effect occurs simply due to the poor design of the siRNA, as siRNA can tolerate several mismatches at the mRNA (imperfect complementarity) without losing gene silencing ability.[65] Under these circumstances, siRNA behaves like a miRNA molecule: it enters the natural miRNA pathway leading to the inhibition or degradation of multiple mRNAs. In certain cases, this type of off-target effect is nearly as efficient as the on-target effect in reducing the protein levels.[66] Another type of off-target effect is not sequence-dependent, but due to the saturation of the RNAi machinery.[61] When synthetic siRNAs (or miRNAs) are introduced into the cells, they compete with the endogenous miRNAs for common proteins such as RISC and other factors. As a result, gene regulation by endogenous miRNAs is perturbed, leading to unpredictable off-target effects.[67]

Reduction of siRNA off-target effects is one of the research priorities in siRNA therapeutics development. Several strategies have been proposed to mitigate such off-target effects. One approach is to use the lowest possible siRNA concentrations, as the off-target effects due to the miRNA-like effect and RNAi machinery saturation are concentration-dependent.[60,67] Pooling of multiple siRNAs targeting the same mRNA is another strategy, which allows the gene silencing effect to be achieved at low concentrations of each siRNA in the pool. As each siRNA has a unique off-target signature, the off-target effects can be selectively reduced.[61,68] However, there is also a risk that siRNA pool may cause more off-target effects than the beneficial effects from on-target activity.[52]

To avoid miRNA-like off-target effect, the logical approach is to reduce complementarity between the seed region (2–7 nucleotides of 5′ end) of siRNA and the 3′UTR of mRNA. Clearly, the seed sequences of miRNA (identified with miRNA databases) should be avoided in siRNA design.[69] In addition, the siRNA should have a low thermodynamic stability of the duplex between the seed region of the guide strand of siRNA and its target mRNA, since a low seed-target duplex stability reduces the capability of siRNA to induce seed-dependent off-target effects.[58,70]

Although the seed region of siRNA is implicated in the miRNA-like off-target effect, only some mRNAs, with this type of sequence complementarity, are silenced by siRNA. By analogy, miRNAs regulate the expression of multiple mRNAs through binding of the seed region to the target 3′UTR, but not all mRNAs with the same degree of sequence complementarity are the targets of a given miRNA.[61] Therefore, it is speculated that several characteristics, other than sequence complementarity of the mRNA, are involved in defining the mRNA as a siRNA and/or miRNA target. However, these characteristics are still poorly understood and the identification of these features could contribute to the design of siRNA with minimal off-target effects. Another way to avoid the occurrence of off-target effects is by chemical modification, which is discussed in more details in section “Chemical modification”.

Avoidance of immune response. Initial studies suggested that long dsRNAs (over 30 nucleotides) could trigger an immune response by activating the IFN pathway.[20] This led to the development of synthetic siRNAs (with smaller number of nucleotides), in the hope to generate therapeutic gene silencing without immunogenic adverse effect.[18] However, it was soon discovered that siRNAs could also activate innate immunity[71,72]; this complication creates another major hurdle to the development of siRNAs as therapeutic agents.

Indeed, siRNAs can cause immune responses in a sequence-independent and sequence-dependent manner. The former involves the PKR and toll-like receptor (TLR) 3 signaling pathways, although they may play only minor roles.[73] The latter is mediated by TLR 7 and TLR 8 on dendritic cells and monocytes, respectively.[74] These receptors are transmembrane receptors present in the endosomes of immune cells. Several immune-stimulatory sequence motifs have been reported. They include (5′ to 3′) “GUCCUUCAA”, “UGUGU”, “UGU”, and “UGGC”.[59,71] Moreover, the presence of U-rich sequences correlates with TLR 7/8 activation.[75] While avoiding these immune-stimulatory sequence motifs could reduce the siRNA immunogenicity, it may be impractical to exclude U from the primary siRNA sequence. Alternatively, stimulation of the TLR 7/8 mediated-immune response could be minimized by the use of delivery agents that exclude siRNA endosomal delivery (*e.g.*, electroporation) or by chemical modification of the immune-stimulatory sequences to render them unrecognizable by TLR.[76] At present, the rules of sequence-dependent immune activation are still poorly understood. Therefore, all therapeutic siRNAs must be carefully tested for any possible immunostimulatory adverse effects.

Design of therapeutic miRNA

Compared with siRNAs, miRNAs have a broader therapeutic application. Over 2,500 human miRNAs have been recorded in the miRBase (version 20 accessed June 2015), a searchable online miRNA database. Since more than 60% of the human protein-coding genes contain at least one conserved miRNA-binding site, together with the presence of numerous nonconserved sites, the majority of protein-coding genes are under the control of miRNAs.[29] The extensive involvement of miRNAs across many human diseases makes them attractive targets for therapeutic strategies, as well as prognostic and predictive biomarkers.[77]

The goal of miRNA replacement therapy using synthetic miRNAs (or miRNA mimics) is to achieve the same biological functions as the endogenous miRNAs. Therefore the synthetic miRNAs should possess the ability to be loaded to RISC and silence the target mRNAs through the natural miRNA signaling pathway. In theory, a single-stranded RNA molecule containing the sequence that is identical to the guide strand of the mature miRNA could be functioned as miRNA mimic. However, the double stranded miRNA containing both guide and passenger strands was found to be 100 to 1,000 times more potent than the single stranded one.[4,14] The double stranded structure can facilitate the proper loading of the RNA molecule into the RISC, thereby enhancing the gene silencing effect. Therefore, designing miRNA mimics with a duplex structure has become the direction of therapeutic development. Synthetic miRNA precursors with longer sequences (from a few extra nucleotides to a full-length pri-miRNA) have also been proposed as therapeutic agents.[78] Since pri-miRNAs require processing in the nucleus, whereas pre-miRNAs and miRNAs do not, different strategies are required for the delivery of different types of miRNA mimics to their cellular targets.[79] Similarly to shRNAs, viral vectors can be used to express miRNAs inside the cells. This review only discusses exogenously delivered, synthetic miRNAs.

The design of therapeutic miRNA is more straightforward than that of siRNA, as the sequence of the former should be almost, if not entirely, identical to the endogenous miRNA of interest. Nevertheless, the development of miRNA therapeutics faces similar hurdles that are encountered by siRNAs. *In vivo* administration of miRNAs can activate the innate immune system via TLR,[80] leading to significant undesirable effects. As the sequence variation for therapeutic miRNA is limited, chemical modification is the major approach to tackle this problem. Furthermore, therapeutic miRNAs also face the barriers of poor stability and inefficient delivery. The

strategies to overcome these barriers are discussed in the following sections.

Chemical modification

RNAs are extremely vulnerable to serum nucleases. Although double-stranded RNA is more resistant to nuclease degradation than single-stranded RNA, naked RNAs in their unmodified forms are degraded rapidly following administration by the abundant nuclease present in the bloodstream, which contributes to a short half-life *in vivo*.[81] Poor stability is one of the major obstacles toward the successful application of siRNAs and miRNAs as therapeutic agents. Chemical modifications of RNA were developed initially to address this issue. In addition, chemical modification of the RNA duplexes can minimize immunogenicity and reduce off-target effects.[82]

A wide variety of RNA modification approaches have been investigated since the development of antisense therapies in the 1980s. The techniques have matured over the years and some are also applicable to siRNAs and miRNAs. Successful chemical modification should not compromise the gene silencing efficiency of these RNA molecules. In order for the modified siRNAs or miRNAs to be compatible with the endogenous silencing pathways and be loaded into the RISC in the correct orientation, a number of factors, including the position and the type of modification, and its effect on the charge of the RNA duplex, needs to be considered.[76] Since the 5′ phosphate, the 5′ proximal part, and the central positions of the guide strand mirror the important areas of the RNA duplex for the interaction with and the action of the RISC and AGO proteins,[83,84] RNA duplexes are less tolerant to chemical modifications at these sites. By contrast, chemical modifications at the entire passenger strand and the 3′ proximal part and 3′ overhang of the guide strand would have the least influence on the specificity and/or function of the RNA.[85]

The major types of chemical modification that are commonly investigated in siRNA and miRNA design include: (i) ribose 2′- OH group modification; (ii) locked and unlocked nucleic acids; and (iii) phosphorothioate (PS) modification (**Figure 3**). Different RNA modification approach may be employed to serve different functions. Combinations of different modification strategies are also commonly used.

Ribose 2′-OH group modification. Modification of the ribose 2′-OH group is the most diverse and also the most popular type of modification in RNA duplex design, as the gene silencing activity of siRNAs or miRNAs does not depend on this group.[86] This strategy involves the substitution of the ribose 2′-OH group with other chemical groups, including 2′-O-methyl (2′-O-Me), 2′-fluoro (2′-F) and 2′-methoxyethyl (2′-O-MOE) (**Figure 3**), and can effectively enhance the stability of the RNA duplex in serum. In particular, substitutions with 2′-O-Me and 2′-F are the two most extensively studied modifications in siRNA. Although these modifications are generally well-tolerated at most siRNA positions, extensive or full modification may lead to significant loss of silencing efficiency.[60] By alternating 2′-O-Me and 2′-F substitutions in a fully substituted siRNA, nuclease-resistant and highly potent modified siRNA can be produced.[87] Bulky substitution such as 2′-O-MOE may enhance nuclease resistance, but is poorly tolerated in terms of activity.[85,88–90]

Apart from enhancing nuclease stability, ribose 2′-OH modifications also reduce immune activation of the RNA duplex.[91] The substitution of the 2′-OH at only the U sites with either 2′-O-Me or 2′-F can abrogate immune responses without affecting siRNA potency.[92] Such modification is believed to render the RNA duplex unrecognizable by TLR 7/8, which is responsible for siRNA-mediated immune response, as U-rich RNA sequences are associated with TLR 7/8 activation.[75] Another approach proposed to reduce TLR 7 activation is to alternate 2′-O-Me modification of the passenger strand thereby preserving the gene silencing potency of the guide strand of the siRNA.[93]

LNA and UNA modifications. Locked nucleic acid (LNA) is a type of chemically modified nucleic acid containing a methylene bridge between the 2′-O and the 4′-C of the sugar to create a stable "locked" ring conformation[94] (**Figure 3**). This modification improves RNA duplex stability by increasing its resistance against nuclease degradation.[95,96] However, multiple LNA modifications may lead to decreased efficacy *in vitro* and *in vivo*.[95,97,98] In order to function properly, siRNA or miRNA must be designed in a way that favors the selection of the intended guide strand by the RISC to minimize off-target effects resulting from the passenger strand being wrongly selected. LNA modification can avoid this type of off-target effect as the modification of the passenger strand at the 5′ end precludes its incorporation into the RISC.[95] Moreover, LNA modification in general can reduce RNA duplex-induced immunogenicity by preventing the immunogenic sequence-motifs from being recognized by TLR 7/8, without affecting its silencing activity.[99]

In recent years, unlocked nucleic acid (UNA) modification has been introduced to siRNA[100,101] (**Figure 3**). UNA monomers are acyclic derivatives of RNA, lacking the C2′ and C3′-bond of the RNA ribose ring, but structurally similar to unmodified RNA upon incorporation into RNA duplexes. Single UNA modifications are well-tolerated at most tested positions in the passenger and guide strands, exhibiting efficient gene silencing and improved performance and stability both *in vitro* and *in vivo*.[100] However, additional UNA modification, especially in the guide strand, results in reduced silencing efficiency, possibly by destabilizing the siRNA duplex or by interactions with the target mRNA.[100] Furthermore, UNA modification in the seed region of the guide strand can prevent miRNA-like off-target effect without compromising siRNA activity.[101] Overall, results from various studies suggested that UNA modification represent an important modification with potential for future therapeutic RNA design.

Backbone modification. Backbone modifications are commonly used to improve the stability of nucleic acids against nuclease resistance by substituting the phosphodiester backbone linkages with other types of linkage. Among those, phosphorothioate (PS) modification is the most widely used strategy, in which one of the nonbridging phosphate oxygen atoms is replaced with a sulfur atom (**Figure 3**).[102] This approach was first described in the development of antisense oligonucleotides, and is very efficient in increasing exonuclease resistance following parenteral administration.[103] The PS modification also promotes plasma protein binding, thereby

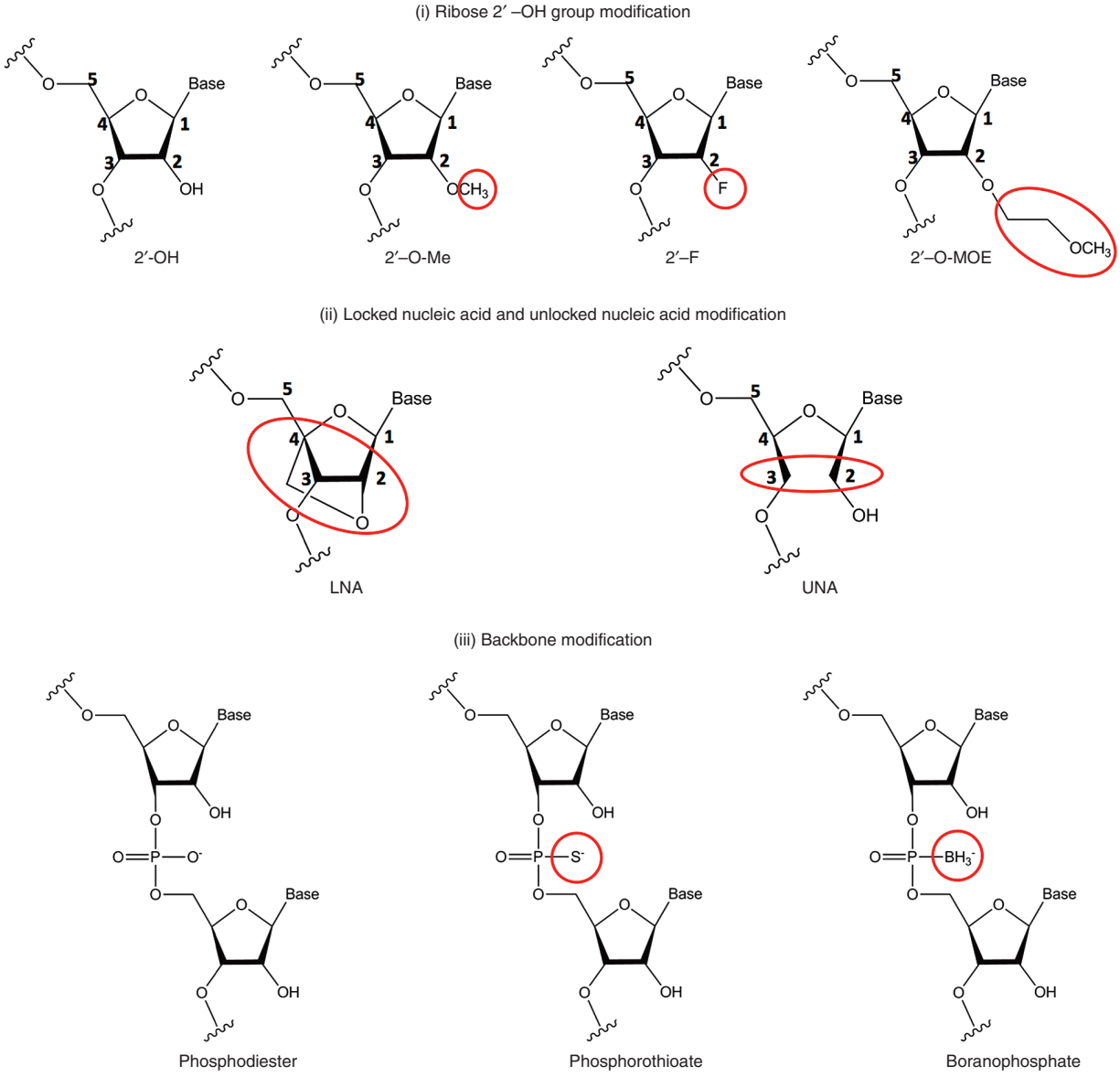

Figure 3 Structures of chemically modified RNA. (i) In the ribose 2' –OH group modification, the 2' –OH group is modified with 2' –O-methyl (2' O-Me), 2' –fluoro (2' –F) or 2' –methoxyethyl (3' –O-MOE). (ii) In locked nucleic acid (LNA) modification, the ribose is locked in a C3' endo conformation by introducing a 2'-O and 4'-C methylene bridge; In unlocked nucleic acid (UNA), the ribose ring is cleaved between 2' -C and 3' -C. (iii) In backbone modification, the phosphodiester backbone linkage is being substituted. The nonbridging phosphate atom is replaced with a sulfur atom to give a phosphorothioate modification, or replaced with a borane (BH_3) moiety to give a boranophosphate modification.

reducing clearance by glomerular filtration and urinary excretion, and hence improving the pharmacokinetic profile of nucleotides.[103] This technique was successfully employed in antisense PS drug fomivirsen, which was approved by the Food and Drug Administration in the late 90s.[104]

When this approach was applied to siRNAs, the stability of the modified siRNA was successfully enhanced *in vivo*.[105,106] However, increased toxicity and reduction of gene silencing was also observed.[86,89,105,106] This is probably because siRNAs, unlike antisense oligonucleotides, tolerate only limited modifications to remain RISC-compatible. It has been suggested that PS modification at the center of the duplex, especially at the scissile phosphate position, impairs the activity of the RISC.[49] Therefore, partial PS modification was recommended, together with other types of modifications to enhance exonuclease resistance of siRNAs.[107] This strategy has not been popular due to the limitations mentioned above. However, by carefully controlling the stereochemistry of phosphorothioate siRNA during synthesis, nuclease resistance could be improved without compromising biological activity.[108] Alternatively, boranophosphate modification, which involves the replacement of nonbridging phosphate oxygen atoms with an isoelectronic borane (BH_3) moiety (**Figure 3**),[109] is more nuclease-resistant and less toxic compared to its PS counterparts. Whether or not this modification can retain the biological activity of siRNAs remains to be determined, and the application of this modification in miRNA therapeutics is yet to be studied.

Delivery of siRNA and miRNA Therapeutics

While chemical modification can improve the stability and reduce off-target effects of siRNAs and miRNAs, poor delivery is still a major challenge in translating therapeutic siRNAs and miRNAs into the clinic. Both types of RNA molecules have an intracellular site of action, but their intrinsic properties, including hydrophilic nature, negative charge and high molecular weight (~14–15 kDa), render them poorly permeable across biological membranes. The primary role of a delivery system is to facilitate the cellular uptake of siRNAs or miRNAs to their target sites.[110] A delivery system can also protect the nucleic acids from premature nuclease degradation, thereby reducing the need for chemically modifications, which may affect the specificity and functionality of the RNA molecules. Since siRNAs and miRNAs have similar physicochemical properties (double-stranded RNAs with 21–23 nucleotides) and the same intracellular site of actions (both require enzymatic functions of the RISC to be active against the target mRNAs), similar delivery technologies can be applied to both types of RNA molecules.

Viral vectors

Viral vectors encoding shRNAs or miRNAs have been used to trigger RNAi and gene silencing effects.[111] Viruses that are commonly employed for this purpose include lentiviruses,[112–114] adenoviruses,[115–117] and adeno-associated viruses (AAVs).[118–120] They are extremely efficient in transferring the RNA-encoding vectors into the nucleus of mammalian cells to ensure high expression of RNA. Almost 70% of all gene therapy clinical trials have involved the use of viral vectors to deliver nucleic acids because of their high transduction efficiency.[110] Viruses that are used to carry therapeutic RNA are genetically engineered to remove their virulence, and their tropism can be altered by genetic manipulation of the viral capsid for targeting to specific cell types.[27] In addition, long-term expression can be achieved by using viruses, such as lentiviruses, that can integrate into the host genome. However, there are serious safety concerns associated with the use of viral vectors, including high immunogenicity (especially in adenoviruses)[121] and the risk of insertional mutagenesis (especially in lentiviruses).[122,123] In addition, low packaging capacity (especially in AAVs)[118] and high production cost have also limited their clinical applications.[124] Therefore, despite their inferior transfection efficiency, nonviral vectors have become attractive alternatives in delivering synthetic siRNAs and miRNAs due to their better safety profile and lower production cost.

Nonviral vectors

Most of the nonviral vectors that have been investigated for RNA delivery are also used to deliver other types of nucleic acids including plasmid DNA and antisense oligonucleotides. Since the development of nonviral vectors has been extensively reviewed, readers who are interested in the design and structure of different types of nonviral delivery systems are referred to a number of recent articles.[79,110,125–127] Polymer-based and lipid-based systems are the two main categories of RNA delivery systems. Apart from the advantages mentioned above (*i.e.*, relatively good safety profile and low production cost), nonviral vectors are highly versatile. They can be easily modified to improve their delivery efficiency, *e.g.*, to achieve site-specific delivery by incorporating targeting ligands, or to improve serum stability and extend the circulation time by PEGylation (attachment of polyethylene glycol (PEG) polymer chains).[110] Despite the effort to develop suitable RNA delivery systems for clinical use, a lack of correlation between *in vitro* and *in vivo* efficacies is observed. It is often reported that a delivery system worked efficiently *in vitro* but failed *in vivo* either due to toxicity problems, poor pharmacokinetic profiles, nonspecific uptake or immune responses.[128] The success of therapeutic siRNAs and miRNAs is highly dependent on the availability of a safe and efficient delivery system. Selected examples of nonviral delivery systems that have been investigated to deliver therapeutic siRNAs and miRNAs in animal and preclinical studies are summarized in **Table 4**.

Polymer-based delivery systems. Cationic polymers can form polyplexes with the negatively charged RNA through electrostatic interactions. The preparation of polyplexes is simple, and the nanosized polyplexes can facilitate cellular uptake through endocytosis. In addition, polymers that exhibit high proton buffering capacity can promote endosomal escape, thereby avoiding endosomal-lysosomal RNA degradation. Synthetic polyethylenimine (PEI), which has an extensive pH buffering capacity, is one of the early generation polymers studied for nucleic acid delivery.[129] It is the most widely investigated polymer for siRNA and miRNA delivery *in vivo*.[130,131] Because of its high transfection efficiency, PEI is regarded as the gold standard among the nonviral vectors. Apart from PEI, dendrimers, which are highly branched synthetic polymers with well-defined molecular architecture, are also frequently studied for nucleic acid delivery.[132,133] Polyplexes that are formed between dendrimers and nucleic acids are also known as dendriplexes. Poly(amidoamine) (PAMAM)[134–136] and polypropylenimine[137] are cationic dendrimers that have been evaluated for delivering RNA *in vivo*. However, because of their high charge density, cationic PEI and dendrimers are often associated with high toxicity which has limited their clinical use. Therefore, modified versions of PEI or dendrimers are developed to address this issue and to further improve their delivery efficiency.[138–140] Alternatively, natural cationic polymers such as chitosan, which is derived from chitin (commonly found in the exoskeleton of crustaceans), and atelocollagen, which is highly purified protein derived from calf dermis, are considered to be safer options for RNA delivery.[141–145]

Cyclodextrins, the cyclic oligomers of glucose, have been used in pharmaceutical formulations and their long-term effects in humans are well-established.[146] Due to their low toxicity, high stability and lack of immune stimulation, cyclodextrin-based nanoparticles were investigated as a carrier of siRNA.[147,148] Poly(lactic-co-glycolic acid) (PLGA) is a Food and Drug Administration-approved synthetic biodegradable polymer that is widely studied for delivering various types of therapeutic molecules including RNA due to its low toxicity and good safety profile.[126] The rate of drug release can be controlled by the molecular weight and composition of PLGA. Since PLGA is a neutral polymer, it does not form polyplexes with nucleic acids. Instead, RNA can be loaded in PLGA nanoparticles or microparticles.[126] Due to the hydrophilic nature of RNA and the hydrophobic nature of

Table 4 A summary of selected examples of nonviral vectors investigated for delivery of therapeutic siRNAs and miRNAs in animal and preclinical studies in recent years

Delivery system	Disease	miRNA/siRNA	Animal model	Route of administration	Reference
Unmodified PEI					
PEI	Asthma	siRNA targeting IL-13	Mouse sensitized and challenged with ovalbumin	Intravenous	202
	Sepsis	siRNA targeting IL-6 and TNFα	Mouse with polymicrobial sepsis induced by cecal ligation and puncture	Intravenous; intraperitoneal	203
	Colon cancer	miRNA-145; miRNA-33a	Mouse xenograft tumor	Intratumoral; intraperitoneal	204
Modified PEI					
SA-PEI-CNT	Melanoma	siRNA targeting Braf	*In situ* mouse melanoma model	Topical	138
PU-PEI	Lung cancer	miRNA-145	Mouse xenograft tumor	Intratumoral	139
	Gliobastoma	miRNA-145	Mouse xenograft tumor	Intratumoral	140
Dendrimers					
PAMAM	Ovarian cancer	siRNA targeting Akt	Mouse xenograft tumor	Intratumoral	134
	Drug-resistant prostate cancer	siRNA targeting Hsp27	Mouse xenograft tumor	Intratumoral	135
PAMAM-folic acid	Glioma	miRNA-7	Mouse xenograft tumor	Intratumoral; intravenous	136
	Ovarian cancer	siRNA targeting CD44	Mouse xenograft tumor	Intraperitoneal	137
Natural polymers					
Glycol chitosan	Drug-resistant breast cancer	siRNA targeting P-glycoprotein	Mouse xenograft tumor	Intravenous	141
Hyaluronic acid-chitosan	Breast cancer	miRNA-34a	Mouse xenograft tumor	Intravenous	142
Atelocollagen	Prostate cancer	siRNA targeting Bcl-xL	Mouse xenograft tumor	Intravenous	143
	Muscular dystrophy	siRNA targeting Mst	Genetically modified mouse with limb-girdle muscular dystrophy	Intramuscular	144
	Metastatic prostate cancer	miRNA-16	Mouse xenograft tumor	Intravenous	145
PLGA					
PLGA microspheres with PEI	Sarcoma	siRNA targeting VEGF	Mouse xenograft tumor	Intratumoral	149
	Joint inflammation	siRNA targeting FcγRIII	Rat with temporomandibular inflammation induced by Complete Freund's Adjuvant injection	Intra-articular	150
PLGA nanoparticles with PEI	Lung cancer	siRNA targeting STAT3	Mouse with lung cancer induced by carcinogen	Intraperitoneal	151
Other nanoparticles					
Tf-targeted nanoparticles of CDP	Subcutaneous tumor	siRNA targeting RRM2	Mouse with subcutaneous tumor of murine neuroblastoma cells	Intravenous	147
Mesoporous silica nanoparticles with pDMAEMA	Cervical cancer	siRNA targeting PLK1	Mouse xenograft tumor	Intravenous	205
Mesoporous silica nanoparticles with KALA peptide-PEG-PEI	Ovarian cancer	siRNA targeting VEGF	Mouse xenograft tumor	Intravenous	154
Porous silica nanoparticles with GD2 antibody	Neuroblastoma	miRNA-34a	Mouse xenograft tumor	Intravenous	155
Lipoplexes					
Cationic liposomes	Melanoma with lung metastasis	siRNA targeting Mcl1	Mouse with lung cancer induced by intravenous injection of murine melanoma or lung carcinoma cells	Intrapulmonary	206
PEG-cationic liposomes	Prostate and pancreatic cancer	siRNA targeting PKN3	Mouse xenograft tumor	Intravenous	190
	Drug-resistant renal cancer	siRNA targeting PLK1	Mouse xenograft tumor	Intravenous	207
RGD peptide -PEG-cationic liposomes	Melanoma with lung metastasis	siRNA targeting c-Myc, MDM2 and VEGF	Mouse with lung cancer induced by intravenous injection of murine melanoma cells	Intravenous	162
Peptides-modified PEG-cationic liposomes	Glioma	siRNA targeting VEGF	Mouse xenograft tumor	Intratumoral; Intravenous	163

Table 4 Continued on next page

Table 4 Continued

Delivery system	Disease	miRNA/siRNA	Animal model	Route of administration	Reference
Cationic liposomes (Lipofectamine™)	Colon cancer	miRNA-143	Mouse xenograft tumor	Intratumoral; Intravenous	208
Cationic liposomes DOTMA/cholesterol/TPGS	Non–small-cell lung cancer	miRNA-29b	Mouse xenograft tumor	Intravenous	160
Neutral lipid emulsion (RNALancerII)	Non–small-cell lung cancer	miRNA-34a, *let-7*	Mouse xenograft tumor	Intravenous	209
Lipid-based nanoparticles (SNALPs, SLNs and LPH nanoparticles)					
SNALP	Ebola infection	siRNA targeting polymerase of Ebola virus	Guinea pigs infected with Ebola virus	Intraperitoneal	210
SLN	Lung cancer	miRNA-34a	Mouse xenograft tumor	Intravenous	211
	Melanoma with lung metastasis	miRNA-34a	Mouse with lung cancer induced by intravenously injection of murine melanoma cells	Intravenous	165
LPH with single chain antibody fragment	Melanoma with lung metastasis	Combined miRNA-34a and siRNA targeting MDM2, c-myc and VEGF	Mouse with lung cancer induced by intravenously injection of murine melanoma cells	Intravenous	167
Lipopolymer					
StA-PEI	Melanoma	siRNA targeting STAT3	Mouse xenograft tumor	Intratumoral	171
DA-PEI	Colorectal cancer	siRNA targeting XIAP	Mouse xenograft tumor	Intratumoral	172
	Myocardial infarction	siRNA targeting RAGE	Rat subjected to ischemic-reperfusion injury by transient coronary artery ligation	Intra-myocardial	173
Cholesterol-PEI	Prostate cancer	siRNA targeting VEGF	Mouse xenograft tumor	Intratumoral	25

Akt, protein kinase B; Bcl-xL, B-cell lymphoma extra large; Braf, v-raf murine sarcoma viral oncogene homolog B; c-Myc, v-myc avian myelocytomatosis viral oncogene homolog, CDP, cyclodextrin-containing polycations; CNT, carbon nanotube; DA, deoxycholic acid; DOTMA, 1,2-di-O-octadecenyl-3-trimethylammonium propane; FcγRIII, immunoglobulin type G cell surface Fc receptor; GD_2, disialoganglioside; Hsp27, heat shock protein 27; IL, interleukin; LPH, lipid protamine hyaluronic; Mcl1, myeloid cell leukemia sequence 1; MDM2, mouse double minute 2 homolog; Mst, myostatin; PAMAM Poly(amidoamine); pDMAEMA, poly-(2-dimethyl-aminoethyl-methacrylate); PEG, polyethylene glycol; PEI, polyethylenimine; PKN3, protein kinase N3; PLGA, poly(lactic-co-glycolic acid); PLK-1, polo-like kinase 1; PU, polyurethane; RAGE, receptor for advanced glycation end-products; RRM2, ribonucleotide reductase subunit M2; SA-PEI, succinated PEI; SLN, solid lipid nanoparticle; SNALP, stabilized nucleic acid lipid particles; STAT3, signal transducer and activator of transcription 3; StA, stearic acid; Tf, transferrin; TGPS, D-α-tocopheryl polyethylene glycol succinate, TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis protein.

PLGA, it is always a challenge to obtain a high loading efficiency. Moreover, the neutral PLGA particles do not promote cellular uptake as effectively as cationic polyplexes. Incorporating small amount of cationic polymers such as PEI into the PLGA nanoparticles can enhance the encapsulation and transfection efficiency. This can also lower the toxicity when compared to the use of cationic polymers alone.[149–151] Silica-based nanoparticles are biocompatible, biodegradable with low toxicity, and have wide biomedical applications.[152] Their high internal pore volume and high capacity for functionalization make them attractive materials for drug delivery,[153] and they have been investigated in recent years for RNA delivery *in vivo*.[154–156]

Lipid-based delivery systems. Similarly to cationic polymers, cationic lipids and liposomes can form lipoplexes with RNA through electrostatic interactions.[157] In general, lipids used for nucleic acid delivery are composed of a cationic head group and a hydrophobic chain. The choice of the head group and the hydrophobic chain may dramatically affect the transfection efficiency and toxicity level of the lipoplexes. Examples of commonly used cationic lipids for nucleic acid delivery include 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP) and 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), which are often used in combination with neutral lipids such as dioleoylphosphatidylethanolamine (DOPE) and cholesterol to enhance transfection efficiency.[157–159] Many of the commercial available transfection reagents such as Lipofectamine, Oligofectamine, DharmaFECT, siPORT, and TransIT-TKO are lipid-based systems and have been frequently used for RNA delivery. Despite their high transfection efficiency *in vitro*, the *in vivo* performance of most lipid-based delivery systems is not satisfactory due to toxicity, nonspecific uptake, and unwanted inflammatory and immune responses.[128,158] Incorporation of PEG is a commonly employed strategy to reduce immunogenicity as well as prolong circulation following systemic delivery, but PEGylation may lead to reduction of cellular uptake. The incorporation of D-α-tocopheryl polyethylene glycol succinate into the delivery system has been reported to overcome this problem. D-α-tocopheryl polyethylene glycol succinate is a water-soluble derivative of natural vitamin E, which is formed by esterification of vitamin E succinate with PEG. It has the advantages of PEG, but also promotes cellular uptake.[160,161] To improve specificity, targeting moieties such as antibodies and small peptides can be employed.[162,163]

Lipid-based nanoparticles with low toxicity and high efficiency are developed to produce a more sophisticated delivery system for RNA. For example, "stabilized nucleic acid lipid particles" in which RNA is encapsulated inside the highly PEGylated liposomes prepared by ethanol dilution method have been proposed.[164] In some studies, RNA is loaded into the cationic solid lipid nanoparticles for

Table 5 A summary of siRNA therapeutics in clinical trials (registered with *clinicaltrials.gov*, last accessed 13 June 2015)

Name	Indications	siRNA target	Phase	Delivery system	Route of administration	Trial ID (reference)
Cancer						
ALN-VSP02	Advanced solid tumors with liver involvement	KSP and VEGF	1, completed	Lipid nanoparticles	Intravenous	NCT01158079; NCT00882180[2]
Atu027	Advanced solid tumor	PKN3	1, ongoing	Liposomal particles (AtuPLEX®)	Intravenous	NCT00938574[3]
	Pancreatic ductal carcinoma		1/2, ongoing			NCT01808638
CALAA-01	Solid tumor	RRM2	1, terminated	Polymer-based targeted nanoparticles	Intravenous	NCT00689065[185]
DCR-MYC (Dicer-substrate siRNA)	Solid tumor, multiple myeloma, non-Hodgkin's lymphomas	MYC oncogene	1, ongoing	Lipid nanoparticles (EnCore)	Intravenous	NCT02110563
	Hepatocellular carcinoma		1/2, ongoing			NCT02314052
siG12D LODER	Advanced pancreatic cancer	mutated KRAS oncogene	1, completed; 2, ongoing	Biodegradable polymer-based scaffold	Local implantation	NCT01188785; NCT01676259
siRNA-EphA2-DOPC	Advanced cancers	EphA2	1, ongoing	Neutral liposomes	Intravenous	NCT01591356
TKM-080301 (TKM-PLK1)	Primary or secondary liver cancer	PLK1	1, completed	Lipid nanoparticles	Intravenous	NCT01437007
	Neuroendocrine tumors and adrenocortical carcinoma		1/2 ongoing			NCT01262235
Infectious Diseases						
ALN-RSV01	RSV infection	RSV nucleocapsid	2, completed	Naked	Intranasal	NCT00496821[5,212]
	RSV infection in lung transplant patients		2, completed		Nebulization	NCT00658086; NCT01065935[199]
ARC-520	Chronic HBV infection	conserved regions of HBV	1, completed; 2, ongoing	DPC (membrane lytic peptides with cholesterol conjugated siRNA	Intravenous	NCT01872065; NCT02065336; NCT02349126
TKM-100201	Ebola virus infection	Ebola L polymerase, VP24 and VP35	1, terminated	Lipid nanoparticles	Intravenous	NCT01518881
TKM-100802			1, ongoing			NCT02041715
Ocular Conditions						
AGN211745 (Sirna-027)	CNV, AMD	VEGF receptor 1	1/2, completed; II, terminated	Naked	Intravitreal	NCT00363714; NCT00395057
Bamosiran (SYL040012)	Ocular hypertension, glaucoma	ADRB2	1, completed; 1/2 completed	Naked	Topical ocular	NCT00990743; NCT01227291
	Ocular hypertension, open-angle glaucoma		2, completed; 2, ongoing			NCT01739244; NCT02250612
Bevasiranib (Cand5)	Wet AMD	VEGF	2, completed	Naked	Intravitreal	NCT00722384; NCT00259753
	Diabetic macular edema VEGF					NCT00306904
	Wet AMD		3, terminated			NCT00499590
	AMD		3, withdrawn			NCT00557791
PF-04523655 (PF-655)	AMD	RTP801 (hypoxia-inducible factor 1 responsive gene)	1, 2 completed	Naked	Intravitreal	NCT00725686[213]; NCT00713518[214]
	CNV, diabetic retinopathy, diabetic macular edema		2, completed			NCT01445899
	Diabetic retinopathy, diabetic complications		2, terminated			NCT00701181[215]
QPI-1007	Optic atrophy, nonarteritic anterior ischemic optic neuropathy	CASP2	1, completed	Naked	Intravitreal	NCT01064505
SYL1001	Ocular pain, dry eye syndrome	Capsaicin receptor TRPV1	1, completed; 1/2, completed	Naked	Topical ocular	NCT01438281; NCT01776658
Cardiovascular and metabolic diseases						
ALN-PCS02	Hypercholesterolemia	PCSK9	1, complete	Lipid nanoparticles	Intravenous	NCT01437059[216]
ALN-PCSsc			1, ongoing	GalNAc-siRNA conjugation	Subcutaneous	NCT02314442
PRO-040201 (TKM-ApoB)	Hypercholesterolemia	ApoB	1, terminated	Lipid nanoparticles	Intravenous	NCT00927459

Table 5 Continued on next page

Table 5 Continued

Name	Indications	siRNA target	Phase	Delivery system	Route of administration	Trial ID (reference)
Genetic Disorders						
ALN-AT3sc	Hemophilia A and B	AT	1, ongoing	GalNAc-siRNA conjugation	Subcutaneous	NCT02035605[180]
ALN-CC5	PNH	complement component C5	1/2, ongoing	GalNAc-siRNA conjugation	Subcutaneous	NCT02352493
ALN-TTR01	TTR-mediated amyloidosis (FAP)	TTR	1, completed	Lipid nanoparticles	Intravenous	NCT01148953[217]
Patisiran (ALN-TTR02)	TTR-mediated amyloidosis (FAP)	TTR	1, 2 completed	Lipid nanoparticles	Intravenous	NCT01559077[217]; NCT01617967; NCT02053454
			2, 3 ongoing			NCT01961921; NCT01960348
Revusiran (ALN-TTRsc)	TTR-mediated amyloidosis (FAC)	TTR	2, completed	GalNAc-siRNA conjugation	Subcutaneous	NCT01981837
			1, 2, 3, ongoing			NCT01814839; NCT02292186; NCT02319005
TD101	Pachyonychia Congenita	Keratin 6a (N171k mutant)	1, completed	Naked siRNA	Intradermal	NCT00716014[218]
Other diseases						
ND-L02-s0201	Hepatic fibrosis	HSP47	1, completed; 1/2, ongoing	Vitamin A-coupled liposomes	Intravenous	NCT01858935; NCT02227459
QPI-1002 (I5NP)	Acute renal failure, Injury of kidney	p53	1, completed; 1, terminated	Naked siRNA	Intravenous	NCT00554359; NCT00683553
	Prevention of delayed graft function in kidney transplantation		1/2, completed			NCT00802347

ADRB, β-2 adrenergic receptor; AMD, age-related macular degeneration; ApoB, apolipoprotein B; AT, antithrombin; CASP2, Caspase-2; CNV, chloroidal neovascularization; DPC, dynamic polyconjugate; EphA2, ephrin type-A receptor 2; FAC, familial amyloidotic cardiomyopathy; FAP, familial amyloidotic polyneuropathy; GalNac, *N*,Acetylgalactosamine; HBV, hepatitis B virus; HSP47, heat shock protein 47; KRAS, Kirsten rat sarcoma viral oncogene homolog; KSP, kinesin spindle protein; LDL, Low density lipoprotein; MYC, v-myc avian myelocytomatosis viral oncogene homolog, PCSK9, proprotein convertase subtilisin/kexin type 9; PKN3, protein kinase N3; PLK1, polo-like-1; PNH, paroxysmal nocturnal hemoglobinuria; RRM2, ribonucleotide reductase subunit M2; RSV, respiratory syncytial virus; TRPV1, transient receptor potential cation channel subfamily V member 1; TTR, Transthyretin; VEGF, vascular endothelial growth factor; VP, viral protein.

sustained release.[165,166] The low toxic liposome-polycation (or protamine)-hyaluronic acid nanoparticles, in which the negatively charged RNA and hyaluronic acid are complexed with the cationic protamine to form the core of the liposomes, are also being developed for RNA delivery.[167,168]

Lipolyplexes. In recent years, lipolyplexes have emerged as a new generation delivery system for nucleic acids. Typically, such a system is composed of both polymers and lipids in an attempt to address the limitations of polymer-based and lipid-based delivery systems by combining the advantageous characteristics of both.[126,169,170] This approach has been employed for the delivery of RNA *in vivo*. For example, stearic acid was incorporated into the backbone of PEI for siRNA delivery.[171] Modification of PEI with stearic acid led to better transfection efficiency *in vivo* compared to unmodified PEI. Similarly, cholesterol and deoxycholic acid-modified PEI improved the transfection efficiency of siRNA *in vivo*.[172–174]

siRNA AND miRNA therapeutics in clinical studies

The first clinical trial of siRNA therapeutics was initiated in 2004,[175] merely 6 years after the discovery of RNAi. The rapid progress of siRNA advancing into clinical trials is perhaps due to the experience gained during the development of antisense and other nucleic acid-based therapies. To date, around 30 siRNA candidates have reached various stages of clinical trials for the treatment of different diseases (**Table 5**). In comparison, the clinical development of miRNA as therapeutics is lagging behind, with only two miRNA therapeutics, both of which are indicated for the treatment of cancers, being registered in clinical trial to date (**Table 6**). The first miRNA therapeutic trial began in 2013 with the second one starting in early 2015. Although siRNAs share many similarities with miRNAs, the relatively slow progress of miRNA therapeutics could be due to their uncertain mechanism of action and specificity. The diverse potential applications of miRNAs (*e.g.*, as drug target and biomarkers) may also have distracted from their development as therapeutic agents.

However, miRNAs have an advantage over siRNAs as the therapeutics for complex multigenic diseases such as cancers and neurodegenerative disorders, which require modulation of multiple pathways for effective treatment. With the ability to inhibit the expression of a number of target genes, which often work together as a network within the same cellular pathway, a whole disease phenotype can potentially be changed by a single miRNA sequence.[176,177] By contrast, the therapeutic potential of siRNAs is limited by its ability to target only one specific gene. It will be challenging to use siRNAs to modulate complex diseases, although the strategy of employing multiple siRNA sequences in a single formulation has been reported in clinical studies for the treatment of cancers and viral infections.[178] On the other hand, siRNAs are extremely useful for targeting single gene disorders such

Table 6 A summary of miRNA therapeutics in clinical trials (registered with *clinicaltrials.gov*, last accessed 13 June 2015)

Name	Indications	miRNA	Phase	Delivery system	Route of administration	Trial ID
MRX34	Primary liver cancer or liver metastasis from other cancers	miRNA-34a	1, ongoing	Liposomes (SMARTICLES)	Intravenous	NCT01829971
TargomiRs	Malignant pleural mesothelioma; non–small-cell lung cancer	miRNA-16	1, ongoing	Nanoparticles (nonliving bacterial minicells)	Intravenous	NCT02369198

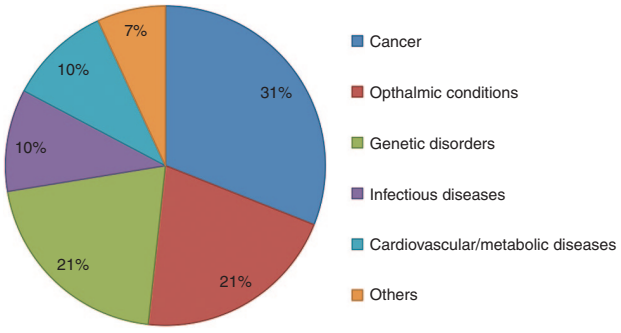

Figure 4 Therapeutic indications of siRNA and miRNA therapeutics.

as hemophilia and hereditary amyloidosis.[179,180] Clinical trials of siRNA and miRNA therapeutics that have been registered with *clinicaltrials.gov* are summarized in **Tables 5** and **6**, respectively. The proportion of conditions addressed by siRNA and miRNA therapeutics in clinical trials is illustrated in **Figure 4**. Since cancer is the only intensively researched condition for which both siRNA and miRNA therapeutics have reached the clinical trial stage, special attention is paid to the discussion of their use in oncology.

Cancer

Cancer is a leading cause of death worldwide. Almost one-third of the siRNA and miRNA based therapeutics in clinical trials are targeted at cancer. Both siRNAs and miRNAs aim to silence cancer-related gene(s) in order to inhibit tumor cell growth, angiogenesis, metastasis and/or drug resistance. Oncogenes, mutated tumor suppressor genes and other genes that contribute to tumor progression are potential targets for gene silencing. The specificity of siRNAs makes them possible to serve as a platform for personalized medicine in cancer therapy.[8] On the other hand, since miRNA therapeutics can target multiple genes, typically in the context of a network, they are efficient in regulating distinct biological cell processes relevant to malignant cell biology. This characteristic makes them particularly attractive in cancer treatment,[77] and may explain why the two miRNA clinical trials to date aim at cancer therapy.

By targeting the mRNA of cell-cycle proteins, tumor cell growth can be inhibited. An overexpression of polo-like kinase 1 (PLK1), a cell-cycle protein that is important in mitosis and cytokinese, is observed in many human tumors, and the inhibition of its activity induces apoptosis and tumor cell death.[181,182] A lipid nanoparticle-based delivery system containing therapeutic siRNA targeting PLK1, TKM-080301, has been developed. It is currently in phase 1/2 clinical trial for the treatment of neuroendocrine and adrenocortical cancers. Ribonucleotide reductase is an enzyme involved in DNA replication. The M2 subunit of ribonucleotide reductase (RRM2) is an established anticancer target.[183,184] Inhibition of RRM2 by siRNAs reduces the growth potential of cancer cells *in vitro* and *in vivo*.[184] CALAA-01 is a siRNA therapeutic targeting RRM2 for the treatment of solid tumors. Adopting a transferrin-receptor targeting cyclodextrin nanoparticle delivery system, CALAA-01 prevents the proliferation of transferrin receptor-expressing tumor cells. A phase 1 study showed that CALAA-01, following systemic administration, silenced the cancer-associated gene by RNAi mechanism in targeted tumor cells.[185] However two patients experienced dose-limiting toxic events in a later trial, possibly due to the formulation problems (mis-folded, aggregated or degraded transferrin); this outcome thus underlines the significance of quality control assays of protein-targeted nanoparticle-based therapeutics.[186]

Other siRNA therapeutics currently undergoing clinical trials include siG12D LODER, which targets the mutated KRAS oncogene for the treatment of locally advanced pancreatic cancer and is delivered by a biodegradable polymer matrix for sustained release; siRNA-EphA2-DOPC, which targets cancer-related EphA2 gene using liposomal delivery for the treatment of advanced solid tumors; and DCR-MYC, a Dicer-substrate siRNA that targets the MYC oncogene carried by lipid nanoparticles for the treatment of various types of cancer.

Angiogenesis is a key process that promotes tumor growth and survival. One of the major regulators of this process is vascular endothelial growth factor (VEGF), which thus is an attractive target for inhibiting tumor angiogenesis.[187] ALN-VSP02 is a dual targeted siRNA therapeutics carried by lipid nanoparticles that suppresses not only the cell-cycle protein kinesin spindle protein (KSP) to promote cell-cycle arrest and eventually cell death,[188] but also VEGF.[2] It is indicated for advanced solid tumors with liver metastasis, and the initial data from a completed phase I trial showed that ALN-VSP02 possessed antitumor activity while being well-tolerated by patients.[2] The siRNA was detected in tumor biopsies following intravenous administration of loaded lipid nanoparticles. Moreover, siRNA-mediated mRNA cleavage in the liver and complete regression of liver metastases was observed in patients with endometrial cancer. Protein kinase N3 (PKN3), a downstream effector of phosphoinositide 3-kinase (PI3K) signaling, is another validated target in cancer. PI3K is only transiently activated after growth factor stimulation in normal cells. Excessive and/or chronic activation of this pathway occurs in many cancer types, and is believed to be involved in the process of metastasis.[189] Inhibition of PKN3 resulted in the significant inhibition of tumor growth and the reduction of lymph node metastases *in vivo*.[190,191] Atu027, a liposomal siRNA targeting PKN3, is currently undergoing a phase 1/2 clinical trial for the treatment of advanced or metastatic

pancreatic tumor. Early results showed that Atu027 was safe in patients with advanced solid tumors, with 41% of patients showed no further progression of tumors after 8 weeks of treatment.[3]

The role of miRNA in cancer has been a focus of research in recent years, evidenced by the number of registered clinical trials evaluating the use of miRNA as biomarkers for patient diagnosis, prognosis, and response to treatment.[40,192] Several miRNAs are upregulated or downregulated in various tumors. They can act either as oncogenes (also known as oncomiRs) or tumor suppressors (tumor suppressor miRNAs),[116] and the latter are employed in miRNA replacement therapy for the treatment of cancer.

MRX34 is a first-in-class cancer therapy and the first synthetic miRNA to enter clinical trials.[193] MRX34 was designed to deliver miRNA-34 mimic by liposomal formulation. Indeed, miRNA-34 is a well-characterized, naturally occurring regulator of tumor suppression,[194] and it is downregulated in many cancers. It inhibits a series of signaling molecules that contribute to cancer processes including cell proliferation, antiapoptosis, metastasis, and chemoresistance.[194] Currently, in phase 1 study, MRX34 is indicated for primary liver cancer or liver metastasis from other solid tumors, and the study is expected to be completed in late 2015. The other miRNA therapeutic that has also reached the clinical trial stage is TargomiRs, which is indicated for malignant pleural mesothelioma and non–small-cell lung cancer. TargomiRs consists of three components, namely a miRNA-16 mimic, a nanoparticle drug delivery system using nonliving bacterial minicells, and an antiepidermal growth factor receptor antibody as a targeting moiety. miRNA-16 is another tumor suppressor.[195] Restoration of miRNA-16 leads to inhibition of tumor-promoting gene transcription and hence tumor growth, as well as sensitization of tumor cells to certain chemotherapeutic agents. The phase 1 clinical study of TargomiRs is expected to be completed in mid-2016.

Although only two miRNA replacement therapies have reached clinical trials, many tumor suppressor miRNAs, such as miRNA-7, miRNA-126, miRNA-143/145, miRNA-200, miRNA-355, and the members of the *let-7* families,[40,192] have been identified with the ability to downregulate oncogenes. Some of these tumor suppressor miRNAs are currently in the preclinical stage and ready to enter phase 1 clinical trials very soon. In terms of safety profile, clinical trials have demonstrated that siRNA therapeutics are generally well-tolerated by the patients. By contrast, the clinical study of miRNA therapeutics is still in its infancy. The potential for any adverse effects may only become apparent after more clinical trials have been carried out. Since a single miRNA can affect multiple target genes, it is also difficult to predict its long-term systemic effect.

Conditions for local treatment

Apart from cancer therapy, clinical trials of siRNA therapeutics against other diseases have also been initiated, including ocular diseases, viral infections, cardiovascular and metabolic diseases, genetic disorders, as well as kidney and renal conditions (**Table 5**). In particular, the use of siRNAs against ocular disease has enjoyed considerable success: around one-fifth of siRNA-based therapeutics in clinical trials are indicated for the treatment of macular degeneration and related eye disorders. The relatively high proportion of siRNA therapeutics indicated for ocular diseases is largely due to the ease of delivery to the target site.[196] The eyes are one of the few organs in the body where successful gene silencing can be achieved by local administration of naked siRNA, thereby minimizing systemic effects, and avoiding toxicity associated with the use of delivery vectors. The most common delivery method is by intravitreal injection of naked siRNA to the posterior segment of the eye, bypassing the corneoscleral barriers. The siRNA can also be topically administered to the ocular surface to treat conditions affecting the anterior segment of the eyes.[196] Despite the promising progress of siRNA therapeutics, miRNAs have yet to enter clinical trials for the treatment of eye disorders, probably due to the uncertainty of the roles of miRNAs in the retina and other ocular tissues.[136,197] With the improvement of knowledge in the field, there is no doubt that the benefits of ocular delivery of siRNAs can be applied to miRNAs for the treatment of other ocular diseases in the future.

Apart from the eyes, the lung is another site in the body where successful RNAi has been achieved in animal studies by local administration of naked siRNA.[198] This effect was also demonstrated clinically by ALN-RSV01, which is a naked siRNA formulation targeting the nucleocapsid of the respiratory syncytial virus (RSV). There is no vaccine for RSV and the only approved therapy (ribavirin) is rarely used due to the risk of teratogenicity and limited antiviral effects. Two phase 2 clinical studies of ALN-RSV01 were completed. ALN-RSV01 was delivered to the lungs of subjects by either intranasal administration or nebulization, and the results of the studies showed that ALN-RSV01 was well-tolerated by patients with promising antiviral effect.[5,199] Although the studies narrowly missed the primary endpoint, they established a unique proof-of-concept for RNAi therapeutics for lung infections by local administration of naked siNRA. However, the cellular uptake mechanism of naked siRNA in the airways is unclear; the presence of lung surfactants along the airways may act as natural carriers that mediate the cellular uptake of siRNA.[200] It is believed that pulmonary delivery could also be applied to other siRNA and miRNA therapeutics for the treatment of other lung diseases.

Conclusions and future prospects

Synthetic siRNAs and miRNAs hold great promises as new classes of therapeutic agents by silencing the gene(s) of interest. They have been studied for the treatment of various human diseases including cancers, viral infections, ocular conditions, genetic disorders, and cardiovascular diseases. The most attractive aspect of siRNA and miRNA therapeutics is their ability to target virtually any gene(s), which may not be possible with small molecules or protein-based drugs. While the therapeutic efficacy of siRNAs and miRNAs has been successfully demonstrated *in vivo*, several technical barriers still need to be overcome in order for these RNA molecules to be used clinically. The experience from antisense and gene therapy has contributed to the rapid progress of siRNAs and miRNAs into clinical studies. In particular, the technologies of chemical modification and delivery of nucleic

acids developed previously can be applied to both siRNAs and miRNAs. While the former possess a high specificity by targeting one single gene, the latter can target multiple related genes, often in the same cellular pathway or process, to generate pronounced therapeutic effect. Currently, the development of siRNAs is advancing ahead of miRNAs, with a larger number of candidates already entered clinical trials, possibly due to the uncertainties of the complex roles of miRNAs during the early years of their discovery. With the recent surge in intensive research concerning miRNAs, it can be expected that significant advance will be made for their future role in therapeutics.

For proper therapeutic use, the sequences of RNA must be carefully designed to avoid any specific or nonspecific unwanted effects and immune responses. The transition from bench to bedside of RNA-based therapy also depends heavily on the availability of a safe, clinically relevant delivery system that can facilitate cellular uptake of the RNA into target tissues/cells and offer protection against nuclease degradation. The use of nonviral vectors including polymer-based or lipid-based delivery systems offer the advantages of better safety profile and lower production cost over viral vectors despite the inferior delivery efficiency.

Among the various diseases being investigated, cancer is currently the major target of siRNA and miRNA therapeutics. While a large number of cancer-related genes have been identified with therapeutic potential, the duration of silencing effect has not been properly investigated or reported, and it may affect the dose interval and length of treatment. The duration of silencing effect after a single dose of siRNAs or miRNAs depends on a number of factors. These include the stability of the RNA molecules, the rate of RNA release from the delivery system, the type of target tissues, as well as the half-life and turnover rate of the target proteins. A good understanding of this area can contribute to the rational design of treatment strategy to improve clinical outcome of siRNA and miRNA therapeutics. PEGylated nanoparticles incorporated with targeting ligands are frequently employed to prolong circulation time and achieve specific targeting to tumor sites following systemic administration. However, the potential toxicity and immunogenicity effects associated with the delivery agents need to be carefully monitored.

Efficient gene silencing can also be achieved with naked RNA, but this may be limited to local administration to organs such as the eyes and the lungs. Nevertheless, chemical modification of the RNA molecule may be required to improve its stability against nuclease activity in the vitreous humor of the eye or in the airway fluid of the lungs. It is expected that by overcoming the delivery barrier, and better understanding of the effects and the duration of gene silencing, siRNAs and miRNAs will become practical therapeutics in the clinic in the near future.

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