

Chapter 2

Synthetic Dicer-Substrate siRNAs as Triggers of RNA Interference

Scott D. Rose and Mark A. Behlke

Abstract The first synthetic oligonucleotides used to suppress gene expression in mammalian cells via RNA interference were 21-nucleotide (nt) RNA duplexes having symmetric 2-nt 3'-overhangs and were designed to mimic the natural products of Dicer processing of long RNA substrates. Synthetic RNA duplexes which are longer than 23-nt length are substrates for processing by Dicer and can show increased potency as artificial triggers of RNA interference, particularly at a low concentration. Longer duplexes, however, can have variable cleavage patterns following Dicer processing which can adversely affect potency. Optimized synthetic Dicer substrates are asymmetric duplexes having a 25-nt passenger strand and a 27-nt guide strand with a single 2-nt 3'-overhang on the guide strand and modified bases at the 3'-end of the passenger strand. This modified design results in predictable patterns of Dicer processing and shows improved activity. The development of this design strategy and use of Dicer-substrate RNAs to trigger gene suppression in a variety of systems will be reviewed in this chapter.

2.1 Introduction

RNA interference (RNAi) is a highly conserved mechanism of gene regulation that extends broadly across phyla [1]. RNAi encompasses two general mechanisms of gene suppression, one where the target mRNA is degraded and a second where protein translation is inhibited [2, 3]. Both routes reduce levels of the protein product made from the targeted gene. Translational suppression is typically mediated by microRNAs (miRNAs), which form imperfect hybrids with the target mRNA [4].

S.D. Rose, PhD • M.A. Behlke, MD, PhD (✉)
Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA, USA
e-mail: mbehlke@idtdna.com

Degradative RNAi, on the other hand, is typically mediated by small interfering RNAs (siRNAs), which form perfect or near-perfect hybrids with the target mRNA [5]. There is substantial “cross talk” between the pathways, and miRNAs can lead to mRNA degradation, and siRNAs can lead to translational suppression [6].

Endogenous siRNAs are symmetric 21-nt RNA duplexes having a 19-nt duplex domain and 2-nt 3'-overhangs that are processed from longer double-stranded (ds) RNAs by the endoribonuclease Dicer [7–9]. Dicer functions as a heterodimer with a second RNA-binding protein, R2D2 in *Drosophila* or TRBP (the human immunodeficiency virus transactivating response RNA-binding protein) in humans [10–12]. Dicer is a large protein with multiple functional domains, including two RNase H family nuclease domains, which perform substrate cleavage, and a PAZ domain, which binds short single-stranded RNA overhangs [13, 14]. Binding of an RNA overhang by the PAZ domain orients the substrate within Dicer. The first nuclease domain is separated from the PAZ domain by a “connector helix,” which determines the distance between the PAZ binding site and the cleavage site, which is 21–22 bases for mammalian Dicer enzymes. Following cleavage, the nascent siRNA remains associated with Dicer and TRBP. An Argonaute (Ago) family protein then associates with the complex, forming a functional RNA-induced silencing complex (RISC) [15, 16]. The siRNA is transferred from Dicer/TRBP to the Ago protein, where the siRNA is converted to single-stranded (ss) form by either cleavage/degradation of one strand or unwinding by a helicase activity [17–19]. The ejected or degraded strand is called “the passenger strand,” and the retained strand is called “the guide strand.” The guide strand directs the sequence specificity of all subsequent gene suppression activity of the complex. Like Dicer, the Ago proteins possess a PAZ domain which binds the 3'-single-stranded overhang of the siRNA and orientates it within the complex [20]. There are four Argonaute proteins in humans that perform different effector functions in RISC [21–24]. In particular, Ago2 is an endoribonuclease which cleaves the target mRNA as directed by sequence complementarity to the siRNA guide strand bound in RISC [25–27] and is the key protein responsible for degradative RNAi.

The first generation of chemically synthesized siRNAs was designed to mimic the natural products of Dicer, i.e., 21-nt RNA duplexes with 2-nt 3'-overhangs [28]. This design remains the dominant form of synthetic siRNAs in use today. During the 10 years since this initial discovery, a variety of artificial designs have been proposed to improve upon some aspect of the RNAi process, which were discussed in a review by Chang and colleagues [29]. This chapter will review development and use of Dicer-substrate siRNAs (DsiRNAs) as a trigger of RNAi.

2.2 Development of Dicer-Substrate siRNA Technology

Dicer is involved in RISC loading, so it is possible that using a synthetic RNA duplex that is a substrate for Dicer and thus engages Dicer prior to RISC assembly may show different properties as a trigger for RNAi rather than an RNA duplex that mimics a Dicer product. This hypothesis was tested in a series of collaborative

experiments performed in the laboratories of John Rossi at the Beckman Research Institute of the City of Hope National Medical Center and at Integrated DNA Technologies [30, 31].

2.2.1 *Characterization of Synthetic Dicer-Substrate siRNAs*

2.2.1.1 Duplex Length and Structure

A series of blunt-ended RNA oligonucleotide duplexes were tested for cleavage in an in vitro dicing assay [30]. The synthetic dsRNAs were incubated with recombinant human Dicer, desalted, and subjected to electrospray ionization (ESI) mass spectrometry. Duplexes as short as 23-nt length were cleaved to 21-nt length, and duplexes from 23- to 30-nt length all showed efficient cleavage. Cleavage efficiency decreased as length was extended to 35, 40, and 45 nts. Thus, synthetic RNA duplexes can function as Dicer substrates, and the optimal length for in vitro dicing was estimated to be in the 25–30-nt range.

The effect of duplex end structure was investigated, and Dicer cleavage occurred whether the duplex had blunt ends, 5'-overhangs, or 3'-overhangs; however, functional potency varied significantly with structure (see below). The effect of end modification (i.e., the addition of non-nucleotide moieties) was investigated by placing a bulky fluorescein group at the 5'-end, 3'-end, or both ends of each strand of the duplex. 5'-modification was well tolerated, but dicing efficiency was markedly reduced by the introduction of a single 3'-modification on either end of the duplex. Cleavage was entirely blocked if the duplex was modified at both 3'-ends. Functional potency in gene knockdown correlated with dicing efficiency. These observations are consistent with a mechanism where the Dicer PAZ domain first binds the substrate RNA at the 3'-end and then cleavage follows; any modification of structure that interferes with 3'-end binding in these very short substrate RNAs disrupts processing.

2.2.1.2 Optimized Design of Dicer-Substrate siRNAs

Functional potency of a series of anti-EGFP RNA duplexes was tested by transfection into EGFP-expressing HEK297 cells [30]. The length of the RNA duplexes was varied from 21 to 30 nts, having either 5'-overhangs, 3'-overhangs, or blunt ends [30]. All of the duplexes tested showed effective suppression of EGFP fluorescence when used at a high concentration (50 nM), but only the longer duplexes showed efficacy when the concentration used was reduced to subnanomolar levels (50–200 pM). At this site, a 27-nt blunt duplex was the most potent compound tested, and the EC_{50} shifted from 20 nM for the 21-nt siRNA to 200 pM for the 27-nt blunt duplex. A prolonged duration of silencing was also observed for the 27-nt duplex, with detectable EGFP suppression increasing from 4 to 10 days; the increased duration of silencing may simply reflect the higher potency of the compound. Significant

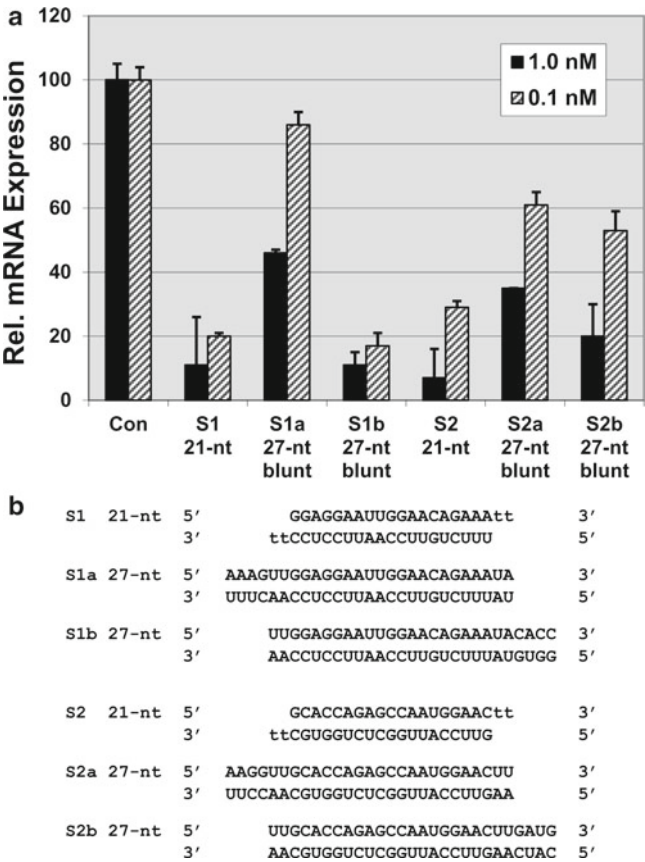


Fig. 2.1 Suppression of *STAT1* expression. The ability of 21-nt siRNAs and 27-nt blunt dsRNA to suppress *STAT1* expression was studied at two sites. (a) *STAT1* (NM_007315)-specific dsRNAs were transfected into HeLa cells at 1 nM or 0.1 nM concentration, and total RNA was isolated 24 h post-transfection. RT-qPCR was performed on the *STAT1* mRNA, and results were normalized to an internal *HPRT1* control. (b) Sequences of the *STAT1*-specific dsRNAs employed are shown with the 27-nt duplexes aligned under the 21-nt siRNA. RNA bases are *uppercase* and DNA bases are *lowercase*

but less dramatic increases in potency were observed at other sites within the *EGFP* gene and also within a set of dsRNAs of varying length that targeted the Sjogren’s syndrome antigen B (*SSB*) gene and the heterogeneous nuclear ribonucleoprotein H1 (*HNRNP1*) gene [30]. Thus, increased potency was observed using Dicer-substrate siRNAs at multiple sites in three different genes in this study.

As the number of sites studied using this “first generation” blunt 27-nt design was expanded, the situation become more complex. Inconsistent performance was sometimes observed between sites such that some duplexes showed higher potency in 27-nt blunt dsRNA form than in 21-nt siRNA form, others showed similar potency between forms, while yet other sites showed higher potency in 21-nt siRNA form. An example of this behavior is shown in Fig. 2.1, where functional potency of

knockdown at two different sites in the *STAT1* gene are compared between a 21-nt siRNA and two blunt 27-nt dsRNAs that overlap the 21-nt sequence. At site 1, one of the two 27-nt blunt dsRNAs showed low potency, while the other showed high potency, similar to a 21-nt siRNA at this site. At site 2, both of the 27-nt dsRNAs showed lower potency than the 21-nt siRNA.

To investigate these seemingly inconsistent results, an assay was developed at Integrated DNA Technologies to study the products of Dicer processing of the synthetic RNA duplexes, hoping to find some correlation between Dicer processing patterns and functional potency. The synthetic dsRNAs were incubated with recombinant human Dicer, desalted, and subjected to electrospray ionization (ESI) mass spectrometry. Using these methods, single Dalton accuracy in mass measurement was achieved, which, in most cases, permitted precise identification of the species produced by Dicer cleavage [31]. It was found that blunt 27-nt substrate dsRNAs were usually cleaved by Dicer into several products. Sometimes as many as five or more different species were observed, which varied in size from 20 to 23 nts. Furthermore, the precise pattern of cleavage could not be readily predicted from sequence. Significant differences in potency are sometimes seen between siRNAs that are shifted by as little as a single base along a target sequence; thus, small differences in dicing patterns could account for large differences in observed potency. These observations also imply that a direct comparison of potency between a blunt 27-nt dsRNA and the “cognate” 21-nt siRNA is really only possible if the dominant 21-nt siRNA produced by Dicer cleavage is identified by a mass spectrometry assay (or other means). Without this information, there is no way to know which of the possible 21-nt siRNAs that could potentially be cleaved from a 27-nt dsRNA substrate is the correct species to employ in a comparative study.

In summary, the mass spectrometry and functional studies discussed above indicated that it was not possible to predict the potency of blunt 27-nt synthetic dsRNAs; sometimes Dicer cleavage resulted in highly potent 21-nt siRNAs from a substrate, and other times low potency species were produced. To improve on these results, a thorough structure–activity relationship (SAR) study was performed where duplexes of different length and design were studied for cleavage patterns and functional potency with the goal of finding a dsRNA substrate that gave predictable results with Dicer processing.

The Dicer-substrate SAR studies identified a design that showed particular promise [31]. The lead compound was an asymmetric dsRNA with a 25-nt sense (passenger) strand and 27-nt antisense (guide) strand. This design has a single 3'-overhang which resides on the guide strand and is blunt at the other end; in addition, two DNA bases were placed at the 3'-end of the passenger strand. Dicer processing of this substrate usually resulted in production of a single 21-nt siRNA species which spanned from the original 3'-overhang in the substrate to a cleavage point 21 bases away. Henceforth, this asymmetric 25/27-nt RNA duplex will be called a “DsiRNA” (optimized Dicer-substrate siRNA). While it is not certain why this design was so effective at encouraging uniform processing, the authors speculated that the single 3'-overhang was bound by the Dicer PAZ domain which positions the substrate at a specific distance from the active endonuclease site, thereby resulting in more uniform processing than was seen using blunt substrates where

there is no 3'-overhang for PAZ binding. Further, the PAZ domain preferentially binds RNA 3'-overhangs, and the inclusion of DNA bases at the 3'-blunt end may further discourage PAZ binding at that end. These ideas are consistent with some of the structural features and biochemistry of Dicer which were subsequently elucidated [13, 14].

Note that an asymmetric 25/27-nt RNA duplex could be designed in two ways, both of which overlap the desired target site and both of which are predicted to produce the same final 21-nt siRNA after Dicer cleavage: one where the passenger strand is 27 nt and one where the guide strand is 27-nt length. Both of these substrate designs were tested at different sites in several genes using the mass spectrometry dicing assay, and it was verified that the same 21-nt siRNA was indeed produced by Dicer processing from these two related but different substrates. For convenience, the asymmetric 25/27-nt RNA duplex with the 27-nt sequence on the guide strand was called the right ("R") form (keeping with the convention that the sense strand is "top," the Dicer PAZ domain binds the single 3'-overhang on the left side of the duplex, and cleavage proceeds to the right "R"). Conversely, the asymmetric 25/27-nt RNA duplex with the 27-nt sequence on the passenger strand was called the left ("L") form (the Dicer PAZ domain binds the single 3'-overhang on the right side of the duplex, and cleavage proceeds to the left "L"). In spite of the fact that both forms result in the same 21-nt daughter siRNA species, very different functional potencies were observed for actual knockdown of a target gene. The "R" form was almost always more potent than the "L" form. An example of this interesting observation is shown in Fig. 2.2, which is reprinted from Rose et al. [31]. A site in EGFP was selected for study, and the potency of a 21-nt siRNA was compared with "L" and "R" form asymmetric 25/27-nt RNA duplexes at the same site. The duplexes were transfected into HEK293 cells that expressed EGFP, and fluorescence was measured 24 h after transfection. The 21-nt siRNA and the "L" form 25/27-nt duplex showed similar potency with around a 70% reduction in EGFP signal seen using 2 nM duplex. In contrast, the "R" form 25/27-nt duplex showed much higher potency, with almost 80% suppression of EGFP fluorescence signal seen at 200 pM; EGFP fluorescence signal was nearly undetectable using a 2 nM concentration of the "R" form duplex.

Using a luciferase-based assay where either a sense target or an antisense target was cloned into the 3'-UTR of firefly luciferase, Rose and colleagues demonstrated that the "L" and "R" forms of the 25/27-nt duplex exhibited differential loading of the guide vs. passenger strands [31]. The 27-nt strand with the 3'-overhang generally shows a relative increase in RISC loading compared with the 25-nt strand. Thus, the "R" form, where the 3'-overhang is on the guide (antisense) strand, generally shows higher functional potency for suppressing expression of the target gene than does the "L" form, which has a loading bias in favor of the passenger strand. These design features are schematically illustrated in Fig. 2.3. Interestingly, 3 years later, a similar effect was reported for 21-nt siRNAs: use of an asymmetric design with a single 3'-overhang on the guide strand improved loading of that strand and increased functional potency [32]. A number of factors influence which strand of an RNA duplex loads into RISC and functions as the guide strand and which strand is

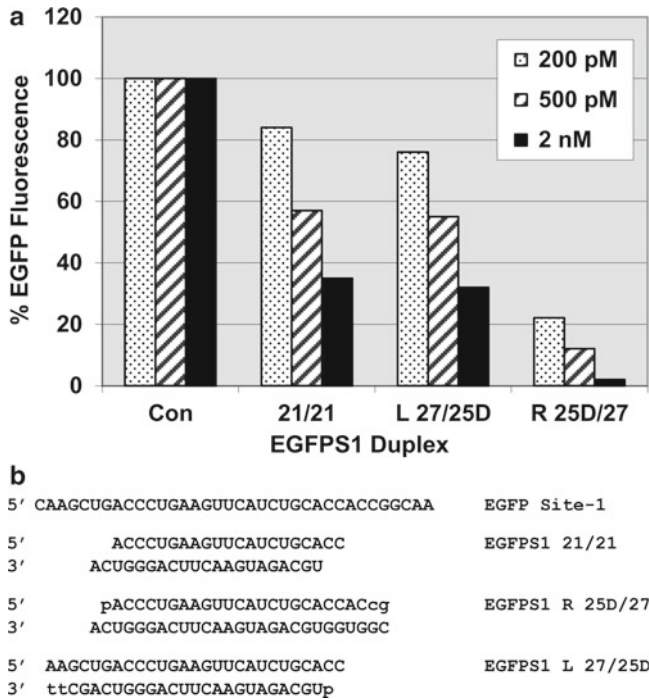


Fig. 2.2 “R” form duplexes are more potent than “L” form duplexes. **(a)** An EGFP expression plasmid was transfected into HEK293 cells. Anti-EGFP or control dsRNAs were transfected 24 h later at the indicated concentrations. At 24 h post-transfection, relative fluorescence was measured setting the control cultures at 100%. **(b)** The EGFP site 1 mRNA target sequence is shown at the top with 21-nt and asymmetric 25/27-nt DsiRNAs aligned below. RNA bases are *uppercase* and DNA bases are *lowercase*. “p” indicates a 5′-phosphate. Reprinted from Rose et al. [31] with permission from Oxford University Press

ejected as the passenger strand. The primary feature contributing to loading bias relates to thermodynamic end asymmetry [33–35]; it now appears that end structure (3′-overhang vs. blunt) also plays a role [31, 32]. Interestingly, a recent report from the Doudna group demonstrated that Dicer is integrally involved in strand selection during RISC formation, lending additional credibility to the functional polarity observed using Dicer-substrate siRNAs in the studies discussed above [36].

2.2.1.3 Functional Potency of DsiRNAs

A study was performed at the Bio-Rad laboratories by Eli Hefner, Teresa Rubio, and colleagues to validate the performance of the newly optimized DsiRNA design [37]. A set of five genes were selected as targets, including *ACTB*, *AKT1*, *RAF1*, *CDK2*, and *TP53*. For each gene, an asymmetric 25/27-nt DsiRNA targeting that gene and the cognate 21-nt siRNA were compared for potency and duration of silencing.

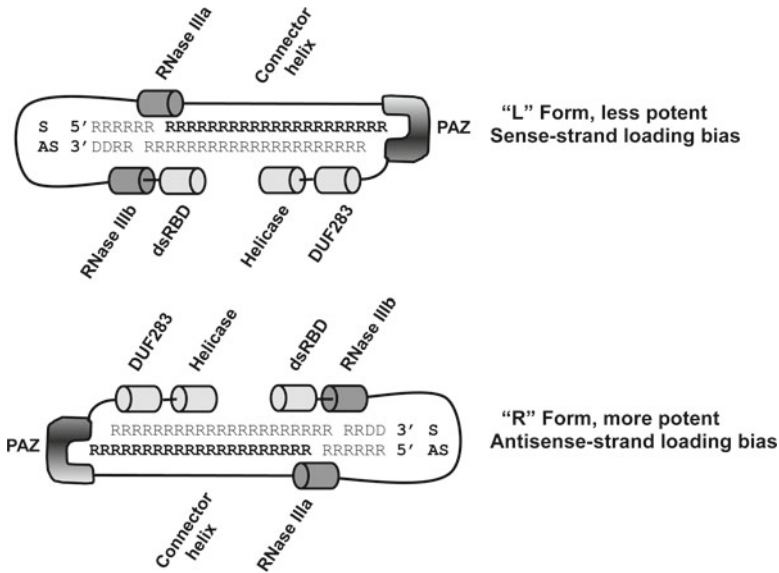


Fig. 2.3 Functional polarity is introduced by Dicer processing. Two forms of DsiRNAs are shown: the "L" form duplex with the single 3'-overhang on the sense strand (S) and the "R" form duplex with the single 3'-overhang on the antisense strand (AS). Sites of Dicer cleavage are indicated by a gap in the sequence; R=RNA, D=DNA. A schematic image of Dicer showing the different functional domains is overlaid on the dsRNA substrate, positioning the RNase III domains at the staggered cut sites and the PAZ domain at the single 3'-overhang. The strand of the cleavage product which is favored for loading into RISC as the "guide strand" is highlighted in **bold**, demonstrating how functional polarity could be introduced by differential positioning of the antisense strand in Dicer between the "L" and "R" forms

Each RNA duplex was transfected into HeLa cells using the siLentFect™ cationic lipid reagent at concentrations of 50 nM, 5 nM, 1 nM, and 100 pM. RNA was extracted 24 h post-transfection, and RT-qPCR was performed to assess relative knockdown of the target mRNA. In four of the five genes studied (*ACTB*, *RAF1*, *CDK2*, and *TP53*), the DsiRNA showed higher potency than the cognate 21-nt siRNA, especially at the lower doses. For the other target (*AKT1*), the DsiRNA and siRNA showed identical potency. The dose response results for the anti-*TP53* DsiRNA and siRNA are shown in Fig. 2.4a, and the sequences employed are shown in Fig. 2.4c.

A comparison of the duration of silencing was also performed. The DsiRNA and siRNA pairs were individually transfected into HeLa cells at 5 nM concentration, and cultures were sampled at days 1, 2, 4, and 6 post-transfection. RNA was extracted, and RT-qPCR was performed to assess relative knockdown of the target mRNA. The results paralleled the dose-response data discussed previously, and, for four of the five genes studied (*ACTB*, *RAF1*, *CDK2*, and *TP53*), the DsiRNAs showed longer duration of silencing than their cognate siRNAs. For the other target

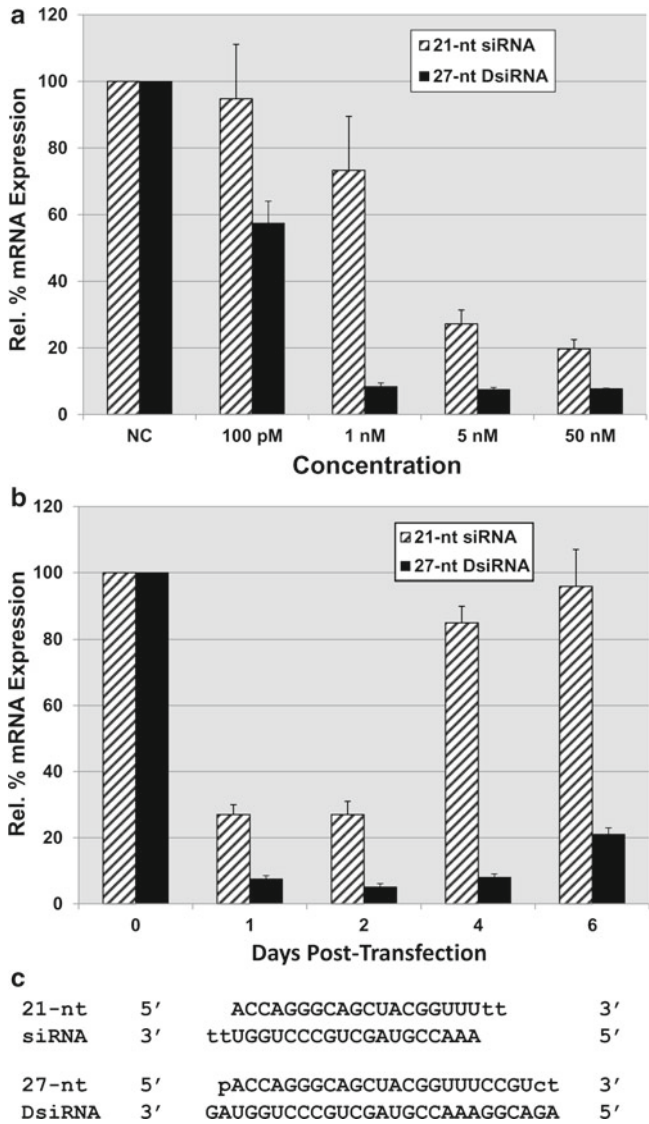


Fig. 2.4 Comparison of potency of an anti-*TP53* DsiRNA and the cognate siRNA. **(a)** An asymmetric 25/27-nt DsiRNA and its cognate 21-nt siRNA targeting *TP53* (NM_000546) were transfected into HeLa cells at the indicated concentrations. RNA was isolated at 24 h post-transfection, and RT-qPCR was performed to measure relative *TP53* expression levels normalized to an internal control gene, *GAPDH*. NC=negative control. **(b)** The same DsiRNA and siRNA were transfected into HeLa cells, and cultures were maintained for 1–6 days as indicated. RNA was isolated, and RT-qPCR was performed to measure *TP53* expression levels as before. **(c)** Sequences of the dsRNAs are shown; RNA bases are *uppercase* and DNA bases are *lowercase*. “p” indicates a 5'-phosphate. Adapted from Hefner et al. [37] with permission from the Association of Biomolecular Resource Facilities (ABRF)

(*AKT1*), the DsiRNA and 21-nt siRNA showed a similar duration of action. It is not thought that the DsiRNA design conveys any specific benefit for duration of action; rather, it appears that duration of action is largely dependent on the potency of the silencing reagent and that for 4/5 of the cases studied here, the DsiRNAs were more potent and therefore showed extended silencing over time. The time course of silencing for the anti-*TP53* DsiRNA and 21-nt siRNA are shown in Fig. 2.4b.

2.2.2 Chemical Modification of DsiRNAs

2.2.2.1 Chemical Modification and Nuclease Stability

Synthetic nucleic acids are readily degraded by nucleases present in serum and in cells. In serum, the primary activity of concern is a 3'-exonuclease [38], whereas in cell extracts endonucleases appear to play a greater role. Fortunately, antisense oligonucleotides (ASOs), siRNAs, and DsiRNAs can be made using chemical modifications which impart nuclease stability as well as improve their safety profiles and general pharmacodynamic properties. Several comprehensive reviews of this topic have recently been published, and readers are referred to these sources for more details [39–41].

It is possible to heavily modify an RNA duplex so that it is almost completely resistant to nuclease attack. Unfortunately, many of the modifications that convey nuclease resistance also reduce the potency of the siRNA, presumably by altering interactions with the various proteins that mediate RNAi in cells, such as Dicer and Argonaute 2 (Ago2). In general, chemical modifications that impart nuclease resistance either involve the internucleoside phosphate bonds or the sugar backbone of the nucleic acid. The phosphorothioate modification (PS) substitutes sulfur for a non-bridging oxygen in the phosphate internucleoside linkage. This conveys relative resistance to many nucleases but can also make the oligonucleotide more “sticky” to proteins and can significantly alter function in undesired ways [42]. While it is common practice to completely modify ASOs with PS bonds, this modification is usually used sparingly in siRNAs. SiRNAs with high PS content can perform poorly, and altered interactions between the RNA and the protein machinery in RISC may be implicated [43]. Selective incorporation of PS linkages near the ends of the duplex and, particularly in the 3'-overhangs, helps protect these vulnerable sites and is a modification strategy commonly used today [40]. Single-stranded RNA domains, such as the siRNA 3'-overhangs, are highly susceptible to degradation. An inverted dT base, PS bonds, or other modifications are often placed at this site.

Modification at the 2'-position of the ribose usually decreases the susceptibility of the neighboring phosphate bonds to nuclease attack, and a wide variety of 2'-modified residues are routinely employed to modify siRNAs. In particular, 2'-*O*-methyl (2'-OMe) RNA is a naturally occurring chemical modification that is found in mammalian tRNAs and rRNAs. This modification is relatively inexpensive to incorporate into synthetic nucleic acids and has no known toxicity. Other

2'-modifications in common use include 2'-fluoro (2'-F) or locked nucleic acids (LNAs), which are a bicyclic nucleic acid with a methylene bridge linking the 2'- and 4'-positions in the ribose ring.

The 2'-OMe modification can be placed in the sense strand, antisense strand, or both strands of a siRNA [44–48]. Complete modification usually results in an inactive siRNA, and use of alternating (or less) 2'-OMe groups is commonly employed. Although not necessary, the 2'-OMe modification is often employed in conjunction with other modifications, such as 2'-F residues. The 2'-F modification is not natural; however, it appears to be generally safe to administer to cells or live animals and can help stabilize siRNAs and improve function [49–52]. In particular, use of 2'-OMe purines with 2'-pyrimidine residues can result in a highly stabilized siRNA with improved performance in vivo [53, 54]. The relative potency of siRNAs having this kind of extensive modification pattern shows sequence dependence and thus may not work effectively at all sites. LNA modifications have an even greater impact on structure and potency of siRNAs and thus are generally used sparingly as modifications. Synthetic oligonucleotides that are heavily LNA modified can show some hepatic toxicity in mice [55], although this effect appears to be sequence dependent and some LNA-modified oligonucleotides are well tolerated [56–58].

The modification strategies discussed above were developed and validated using 21-nt siRNAs. Longer dsRNAs, such as DsiRNAs, appear to naturally show greater resistance to nuclease degradation than short siRNAs [59, 60]. This may in part be due to the higher thermodynamic stability seen for longer duplexes, which may limit the amount of transient single-stranded character in AU-rich regions that are more susceptible to attack by endogenous endoribonucleases, such as RNase A [61]. The same modification strategies employed in 21-nt siRNAs can generally be directly applied to DsiRNAs, except that a small internal domain needs to remain unmodified for Dicer cleavage to occur (the only kind of nuclease attack that is actually desired). It is possible to synthesize DsiRNA duplexes that show high levels of serum stability while retaining the ability to be processed by Dicer. A structural map of the different functional DsiRNA domains is schematically shown in Fig. 2.5. In this figure, the top strand is the passenger strand, and sequence to the left of the Dicer cleavage site comprises the final 21-nt siRNA. This region can generally be modified in ways similar to other synthetic 21-nt siRNAs, as described above. Sequence to the right of the Dicer cleavage site can also be modified; note that this short sequence is discarded and is not part of the final product that enters RISC. As shown in Fig. 2.5, the favored site to add bulky end modifications to a DsiRNA is at the 3'-end of the passenger strand (labeled “ligand”); modifications can also be added to the 5'-end of the guide strand. Using this approach, a bulky modifying group such as a fluorescent dye or ligand that may aid delivery (such as cholesterol, cell-penetrating peptides, etc.) can be attached to the RNA duplex in a way that it is “disposable”; any group connected to this end is cleaved off the DsiRNA and discarded and so does not remain on the mature siRNA and thus does not enter RISC and cannot affect RISC loading.

Collingwood and colleagues reported a systematic survey of various modification patterns in DsiRNAs, focusing on the use of the 2'-OMe and 2'-F modifications [62]. The initial survey was performed at a site in the human *STAT1* gene (corre-

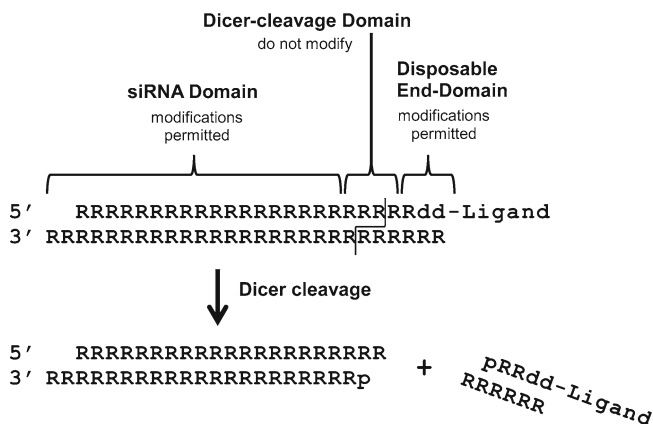


Fig. 2.5 Schematic of DsiRNA domains for chemical modification. An asymmetric 25/27-nt DsiRNA is shown on the *top line* with the domains suitable for chemical modification indicated by *brackets*. A small interior domain offset to the *right of center* is *not bracketed*, which is the site of Dicer cleavage; this region should remain unmodified. The preferred attachment site of ligands is shown on the *right*, connecting to the 3'-end of the passenger strand. The 21-nt siRNA that results from Dicer processing is shown along with the discarded cleavage fragment. RNA bases are *uppercase* and DNA bases are *lowercase*; “p” indicates a 5'-phosphate

sponding to the STAT1 site 2 shown in Fig. 2.1). Like 21-nt siRNAs, some of the more heavily modified duplexes showed significant impairment of functional potency, while less highly modified patterns remained potent. 2'-OMe modification of the sense and/or antisense strands in the “siRNA Domain” (Fig. 2.5) in an alternating pattern was particularly effective, and this approach to modification was also shown to work well at additional sites in the human *HPRT1*, mouse *F3*, and *EGFP* genes. The mass spectrometry dicing assay was used to examine processing of a set of anti-*HPRT1*-modified DsiRNAs, and as long as the modifications did not extend into the Dicer cleavage domain, the expected siRNA products were made following in vitro dicing. Further, DsiRNAs modified with only 11 2'-OMe residues on the antisense strand showed a significant improvement in stability when incubated in serum compared to unmodified 21-nt siRNAs or 25/27-nt DsiRNAs. While this simple modification pattern is often effective, it can impair potency in a sequence-specific fashion at some sites, so additional optimization of the precise placement of modified bases can be beneficial.

Nishina and colleagues described use of a modified asymmetric 27/29-nt DsiRNA to suppress *Apob* expression in mouse liver [63]. 2'-OMe RNA residues and PS bonds were placed at optimized locations in the sense and antisense strands, avoiding modification of the Dicer cleavage domain. Vitamin E (α -tocopherol) was attached to the 5'-end of the antisense strand via a phosphate linkage. The sequence and modification pattern of this compound are shown in Fig. 2.6. The modification pattern employed six 2'-OMe residues and a single PS bond in the sense strand and nine 2'-OMe residues with five PS bonds in the antisense strand and achieved sufficient stability to be used via direct naked intravenous injection in mice. The

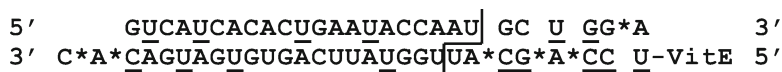


Fig. 2.6 Chemical modification pattern of an anti-*Apob* DsiRNA. Sequence of a modified DsiRNA targeting the *Apob* gene (NM_009693) is shown [63]. RNA bases are *uppercase* and 2'-OMe RNA bases are *underlined*; “*asterisk*” indicates a PS (phosphorothioate) internucleotide linkage. Vitamin E (α -tocopherol) was attached to the 5'-end of the antisense strand via a phosphate linkage. The sites of Dicer cleavage are indicated

vitamin E group is a lipid-soluble antioxidant vitamin that can gain entry to all mammalian cells. Similar to cholesterol, vitamin E associates with serum lipoproteins; the SCARB1 scavenger receptor and LDL receptor are involved in cellular uptake [64]. By attaching vitamin E to the 5'-end of the antisense strand of a DsiRNA, the ligand became part of the disposable “end domain” of the DsiRNA, which is cleaved off of the RNA duplex and does not remain attached to the mature siRNA and, therefore, poses no risk to interfere with RISC entry. *Apob* mRNA levels were reduced by 80% at a dose of 32 mg/kg using this approach.

Kubo and colleagues studied modification of the ends of DsiRNAs with an aliphatic amino modifier and found that addition of this simple group could improve stability and potency of an otherwise unmodified duplex [65]. In particular, modification at the 3'-end of the sense strand had favorable effects. Consistent with the earlier finding reported by Kim [30], placement of a single modifying group at the 3'-end of the antisense strand impaired Dicer processing, while modification of both of the 3'-ends prevented Dicer cleavage. This group also conjugated palmitic acid (C16) at the 3'-end of the sense strand, a location that permits removal of the ligand so the modifier is not present in the mature 21-nt siRNA following Dicer processing (see Fig. 2.5). The 3'-palmitic acid modification led to additional stabilization of the duplex, and an otherwise unmodified DsiRNA survived 48-h incubation in 10% fetal calf serum. Addition of the C16 aliphatic chain also promoted naked delivery of the modified DsiRNA to HeLa cells in tissue culture when used at a relatively high dose (200 nM).

2.2.2.2 Chemical Modification and Immune Stimulation

The mammalian innate immune system employs a fixed repertoire of receptors which recognize structures that are usually associated with pathogens (pathogen-associated molecular patterns or PAMPs), such as bacterial flagella or bacterial lipopolysaccharide. The innate immune system is also capable of recognizing nucleic acids, including single-stranded and double-stranded RNA (ssRNA, dsRNA); this system probably evolved as a fast-response pathway to viral infection. Several members of the Toll-like receptor family (TLRs) recognize RNA, such as TLR3 which binds dsRNA and TLR7 and TLR8 which bind ssRNA. TLR3, 7, and 8 primarily reside in endosomal compartments which limits their contact with endogenous RNAs, helping to limit the risk of an autoimmune response. Further, these receptors preferentially recognize unmodified RNA. RNAs bearing several

modifications that are common in mammalian cells, such as 2'-OMe RNA, pseudouridine, and others, typically help the RNA molecule avoid detection [66]. In fact, 2'-OMe RNA is a competitive inhibitor of TLR7 and can block recognition of unmodified RNAs by this receptor, even in *trans* [67]. Thus, employing the 2'-OMe RNA modification, even in only one strand, can block recognition of a siRNA by TLR7. Other 2'-modifications, such as 2'-F and LNA, also help avoid immune detection. Additional sensors exist in the cytoplasm that can also trigger a response to foreign RNAs, such as PKR, OAS, RIG-I, and MDA5. Many mammalian cells that have adapted to growth in cell culture have lost the ability to respond to foreign nucleic acids; however, the risk of triggering an immune response in vivo, where all cell types are present, is quite high. Strategies to evade immune detection are essential to all methods that use synthetic nucleic acids in mammals, including siRNAs. The reader is referred to some excellent recent reviews on this topic for more information [68, 69].

Like all synthetic RNAs, DsiRNAs can trigger an unwanted immune response when introduced into mammalian cells. In fact, long RNAs are generally more potent at stimulating an immune response than are short RNAs. Thus, there was initially some concern that dsRNAs in the 27-nt length range would present a greater risk for triggering immune responses than traditional short siRNAs. In support of this idea, Reynolds and colleagues studied the ability of different dsRNAs to trigger immune responses in a variety of cell types and found that cell type, the length of the synthetic RNA, and method of delivery were all contributing factors to immune stimulation and other off-target effects [70, 71]. Reynolds studied blunt 27-nt dsRNAs and found that these structures showed a significantly higher propensity to trigger immune responses in several cell types than 21-nt siRNAs (with a 2-base 3'-overhang on both ends). However, it later became evident that end structure is an additional important feature to consider in triggering immune responses. Marques and colleagues reported that transfection of 27-nt dsRNAs with 3'-overhangs on both ends into T98G glioblastoma cells did not trigger an immune response while use of blunt 27-nt dsRNAs resulted in a brisk immune response [72]. The optimized DsiRNA design (asymmetric 25/27 nt with a single 3'-overhang on one side and a blunt end with two 3'-DNA bases on the other end) was a relatively weak immune trigger. Collingwood and colleagues further demonstrated that addition of 2'-OMe residues to the optimized asymmetric 25/27-nt DsiRNA design prevented stimulation of interferon- α (IFN- α) secretion from human peripheral blood mononuclear cells (PBMCs) [62]. PBMCs are considered a sensitive cell population to employ when testing the potential for a synthetic nucleic acid to trigger an immune response as PBMCs are a mixed population of cells which comprise a wide variety of normal immune-competent white blood cells. Using cationic lipid-mediated transfection, unmodified 21-nt siRNAs and 25/27-nt DsiRNAs both stimulated significant levels of IFN- α secretion in human PBMCs, whereas 2'-OMe-modified versions of the same sequences did not. Thus, the combined use of chemical modification and optimized end structure enabled safe use of 25/27-nt DsiRNAs.

2.3 Studies Using DsiRNAs In Vivo

Many studies have been published that employ siRNAs in animals. The scientific issues involved in conducting in vivo RNAi studies in mammals and overviews of significant articles in this rapidly growing field have been well covered in recent reviews [73, 74]. Some recent successes using DsiRNAs in vivo will be discussed below. The in vivo studies discussed herein are summarized in Table 2.1.

Like all classes of synthetic dsRNAs, delivery is the most significant hurdle to widespread use of DsiRNAs in vivo. While the systemic effects associated with intravenous injection are often desirable, local administration is usually easier to perform and suffers from fewer toxicity issues. Several studies have been performed with DsiRNAs using local delivery, including intraperitoneal (IP) and intrathecal (IT) routes of administration. Amarzguioui and colleagues described methods to deliver DsiRNAs using an inexpensive commercially available cationic lipid in mice via IP injection [85]. It was observed that the lipid TransIT-TKO™ was particularly effective in delivering DsiRNAs into macrophages, both in cell culture and in vivo. The peritoneal cavity is a reservoir for monocyte lineage cells, and IP injection offers an opportunity to easily introduce DsiRNA reagents into this cell population via local administration. Immune cells like macrophages are mobile: the transfected macrophages can be recruited to sites of inflammation outside of the peritoneum, thereby achieving systemic effects using a local administration strategy.

Lundberg, Cantin, and colleagues employed this strategy to suppress production of tumor necrosis factor alpha (TNF- α) by macrophages during acute herpes simplex virus (HSV) infection in the mouse central nervous system (CNS) [75]. Studies were performed in C57BL/6, a strain of mice that normally survives herpes encephalitis (HSE). TNF- α is an important factor in the resistance of this strain to HSE, and C57BL/6 mice that are double knockouts for the two known TNF receptors (p55^{-/-}, p75^{-/-}) show increased fatality following HSE. Mice were administered the anti-*Tnf* DsiRNA DsiRNA complexed with the cationic lipid TransIT-TKO™ as a single 2 μ g dose (0.1 mg/kg) in 200 μ L volume by IP injection immediately prior to infection with HSV, then received five additional doses of 4 μ g (0.2 mg/kg) on days 1, 2, 4, 6, and 8 [for a total of six doses with a total cumulative dose of 22 μ g (or 1.1 mg/kg) of the DsiRNA]. Surprisingly, suppression of TNF- α production using an anti-*Tnf* DsiRNA resulted in significantly increased mortality rates, suggesting that the presence of TNF- α somehow alters the pathophysiology of HSE even in the absence of the two known TNF receptors. The mechanism of this unexpected observation remains under investigation. In this case, it is not believed the IP administered DsiRNA directly entered the mouse CNS. Rather, the DsiRNA were taken up by macrophages, which were subsequently recruited by the inflammatory process ongoing in the brain due to the HSV infection, thereby achieving CNS effects from IP administration of a compound that normally cannot cross the blood brain barrier (BBB).

Lundberg, Cantin, and colleagues later used this same system (IP injection of anti-*Tnf* DsiRNA complexed with TransIT-TKO™ to transfect macrophages) to alter the course of fatal hepatic necrosis during endotoxin-induced “toxic shock”

Table 2.1 Studies employing DsiRNAs in vivo

Citation	Delivery route	Carrier	Target	System
Lundberg [75]	IP	Lipid	<i>Tnf</i>	Mouse, CNS herpes simplex encephalitis
Lundberg [76]	IP	Lipid	<i>Tnf</i>	Mouse liver, endotoxin shock
Howard [77]	IP	Chitosan	<i>Tnf</i>	Mouse, collagen-induced arthritis
Nawroth [78]	IP	Chitosan	<i>Tnf</i>	Mouse, radiation-induced fibrosis
Dore-Savard [79]	IT	Lipid	<i>Ntsr2</i>	Rat CNS, nociception
LaCroix-Fralish [80]	IT	Lipid	<i>Atp1b3</i>	Mouse CNS, nociception
Sato [81]	IV	Vitamin A targeted liposome	<i>Serpinh1</i>	Rat, acute hepatic cirrhosis
Kortylewski [82]	IV	CpG oligo conjugate	<i>Stat3</i>	Mouse, metastatic melanoma
Nishina [63]	IV	Vitamin E conjugate	<i>Apob</i>	Mouse liver, cholesterol metabolism
Neff [83]	IV	Aptamer conjugate	HIV <i>tat/rev</i>	Humanized mouse, HIV
Zhou [84]	IV	PAMAM dendrimer	HIV <i>tat/rev</i> , <i>CD4</i> , <i>TMPO3</i>	Humanized mouse, HIV

Studies are shown in the order presented in the text. Routes of administration, *IP* intraperitoneal, *IT* intrathecal, *IV* intravenous

[76]. Bacterial lipopolysaccharide (LPS) binds the receptor TLR4 and triggers release of a number of pro-inflammatory cytokines, including large amounts of TNF- α by macrophages. The “cytokine storm” induced by LPS can lead to hypotension, shock, and death. One early sign of the severity of this shock state is hepatic necrosis. Mice were administered the anti-*Tnf* DsiRNA complexed with TransIT-TKOTM as a single 5 μ g dose (0.25 mg/kg) in 200 μ L volume by IP injection immediately prior to injection of the low dose LPS/D-GalN cocktail. Downregulation of TNF- α production by peritoneal mononuclear cells following this treatment reduced the magnitude of liver damage and delayed mortality. This study again demonstrated the ability to achieve systemic effects in an inflammatory disease state using IP administration of a DsiRNA targeting a cytokine expressed in macrophages.

Howard, Kijems, and colleagues employed this same strategy to treat collagen-induced arthritis (CIA) in mice [77]. In this case, a novel chitosan-based nanoparticle delivery system was employed [86]. The chitosan nanoparticles employed in this study exhibit a net positive charge that may lead to serum-induced aggregation after IV injection but are ideal for use with IP injection and are efficiently taken up by peritoneal macrophages. TNF- α secreted by macrophages is an important factor in the development and pathophysiology of inflammatory arthritis, and anti-TNF- α antibodies are presently employed to treat rheumatoid arthritis. Mice were immunized with an anthrogein-CIA collagen emulsion and displayed the onset of arthritis within 28–35 days postinjection, at which point DsiRNA treatments were initiated. Mice received either 5 μ g of chitosan-complexed unmodified anti-*Tnf* DsiRNAs or control DsiRNAs (0.25 mg/kg) or 2.5 μ g of chitosan-complexed 2'-OMe-modified anti-*Tnf* DsiRNA (0.125 mg/kg) in 200 μ L volume by IP injection on days 1, 3, 5, 7, and 9. Arthritis scores were measured, and joints were examined by histopathology. Suppression of TNF- α by IP administration of an anti-*Tnf* DsiRNA reduced joint swelling, and mice receiving this treatment showed minimal erosion of the articular surfaces of affected joints, while control mice showed severe cartilage destruction. Overall, use of 2'-OMe-modified DsiRNAs showed the most effective biological responses.

The same group used the chitosan-based nanoparticle delivery system to treat radiation-induced fibrosis (RIF) in mice using the IP administration route [78]. RIF arises from damage and scarring within tissue exposed to high levels of ionizing radiation and is characterized by reduced mobility, strictures, pain, and even tissue necrosis. Morbidity from RIF can limit the dose of radiation that can be administered in the treatment of various cancers, and methods to reduce or prevent RIF would have significant value. There is some evidence to suggest TNF- α plays a potentially significant role in this disease. The hind limbs of mice were given a single 45-Gy dose of gamma radiation. Chitosan-complexed unmodified control or anti-*Tnf* DsiRNAs were administered at a dose of 5 μ g (0.25 mg/kg) in 200 μ L volume by IP injection for a varying periods of time, given 2 days prior to irradiation, 1 day post-irradiation, and biweekly thereafter. Mice receiving anti-*Tnf* DsiRNA therapy for <3 weeks all developed RIF, whereas none of the mice that received continuous therapy for 3 weeks or longer developed RIF. All mice that received the scrambled control DsiRNA developed RIF. This work suggests

that intervention of TNF- α production is a valid therapeutic approach to prevent the development of RIF during antitumor therapy.

Two groups have employed the cationic lipid iFECT™ to deliver DsiRNA into the CNS via IT injection. The BBB presents an obstacle for delivery of large molecule compounds to the CNS using IV injection; direct injection into the CNS is the most direct solution to this problem. Dore-Savard and colleagues used anti-*Ntsr2* DsiRNAs to study the function of neurotensin receptor 2 (a GPCR) in thermal nociception in rats [79]. *Ntsr2* mediates analgesia, and administration of the synthetic neurotensin-2 agonist JMV-431 reduces perception of pain. Downregulation of the *Ntsr2* receptor by anti-*Ntsr2* DsiRNAs should block the expected analgesic effects following JMV-431 administration. The anti-*Ntsr2* DsiRNAs were given as two IT injections at spinal levels L5/6 1 day apart using a dose of 1 μ g in iFECT™ (0.005 mg/kg). The neurotensin agonist JMV-431 was administered daily at days 1–4 following the DsiRNA injections, and the rats were studied for nociceptive behavior using the thermal tail-flick test. Analgesic effects from the JMV-431 were absent on days 1 and 2 and slowly returned to baseline over the next several days. Reductions in both *Ntsr2* mRNA and protein were observed, consistent with an RNAi-mediated suppression of *Ntsr2* gene expression.

LaCroix-Fralish and colleagues studied function of the $\beta 3$ subunit of the Na⁺–K⁺–ATPase pump (*Atp1b3*) in the pain response to formalin footpad burns in mice [80]. The mouse strain C57BL/6 shows a higher pain response in this test compared to the A/J strain. Traditional genetic approaches and QTL analysis had previously implicated the *Atp1b3* gene as possibly being involved in this interstrain variability; however, no role for this gene in nociception had ever been demonstrated. Anti-*Atp1b3* DsiRNAs formulated in iFECT™ were given by IT injection at a dose of 0.5 μ g (0.025 mg/kg) once a day for 3 days, and the mice were, thereafter, studied for pain responses to formalin footpad injection. Mice given the anti-*Atp1b3* DsiRNA showed similar pain responses between strains. Within the control DsiRNA cohort, the C57BL/6 mice showed higher sensitivity than the A/J mice (i.e., a wild-type response was seen). These results confirmed a role for *Atp1b3* in nociception in the mouse spinal cord.

Sato and colleagues used symmetric 27/27-nt Dicer-substrate siRNAs (with a 2-nt 3'-overhang on both ends) to treat hepatic cirrhosis in rats using a targeted liposomal delivery system [81]. It is thought that collagen production by the hepatic stellate cells is crucial to development of hepatic cirrhosis following acute or chronic injury. Targeted liposomes were made using the Lipotrust lipid reagent system conjugated to vitamin A as a means to facilitate delivery to the stellate cells. Symmetric Dicer-substrate siRNAs targeting the collagen chaperone heat shock protein 47 gene (*Serpinh1*, gp46) were administered IV at doses up to 0.75 mg/kg three times weekly. This treatment, in contrast to control reagents, reduced collagen production by the stellate cells and prevented fibrosis in several different models of acute cirrhosis, including bile duct ligation and chemical injury with dimethylnitrosamine or carbon tetrachloride.

Kortylewski and colleagues described use of a novel method to improve delivery of DsiRNAs to cells expressing TLR9 known to bind “CpG-motif” DNA (an unmethylated cytosine–guanine dinucleotide) [82]. TLR9 is expressed by some immune

cells, such as plasmacytoid dendritic cells and B cells. Mice also express TLR9 on myeloid dendritic cells and monocytes. Like the RNA-binding receptors TLR3, 7, and 8, TLR9 primarily resides in the endosomal compartment. A DsiRNA targeting Stat3 was conjugated to a phosphorothioate-modified single-stranded DNA sequence termed CpG 1668, a known potent class B TLR9 agonist [87]. Stat3 expression is increased in some tumors where it promotes cell division and tumor growth. It also reduces local immune responses to the tumor, thereby further aiding tumor survival. Thus, suppressing Stat3 could be beneficial and reduce tumor cell survival by several mechanisms. The anti-Stat3+CpG DsiRNAs were taken up by cells into the endosomal compartment via a TLR9-independent mechanism. Cells lacking TLR9 did not show any gene suppression effects. For cells expressing TLR9, some fraction of the material escaped into the cytoplasm and led to suppression of Stat3. This reagent was used to treat mice bearing subcutaneous implants of syngeneic B16 melanoma cells. Direct peritumoral local injections of the anti-Stat3+CpG DsiRNAs were performed in the tumor nodules at a dose of 0.78 nmol per injection (20 μ g, or \sim 1 mg/kg). After three daily injections, significant regression of the B16 tumor implants was observed. Next, B16 tumor cells were injected IV, and the growth of pulmonary nodules was measured over time (an established method to mimic the behavior of metastatic melanoma). The anti-Stat3+CpG DsiRNAs were administered IV at a dose of 0.78 nmol per injection (20 μ g, or \sim 1 mg/kg). The intravenous injections were given every other day for 2 weeks and led to a significant reduction of both the size and number of B16 implants detectable in the lungs of mice receiving the anti-Stat3 therapy but not in the control mice. Another example for ligand-mediated delivery, Nishina and colleagues used direct IV injection of a highly modified anti-Apob DsiRNA conjugated to vitamin E (α -tocopherol) to reduce expression of apolipoprotein B in liver and reduce serum cholesterol levels [63]. Refer to discussion in Sect. 2.2.2.1 for additional details.

Aptamers are highly structured nucleic acid molecules which form conformations that can bind a target ligand with high affinity and specificity, much like antibodies. Aptamers can be conjugated to other compounds to facilitate targeted delivery. For more information, see recent review articles by Thiel [88], Zhou [89], and Syed [90]. Aptamers have been employed to facilitate delivery of DsiRNAs both in vitro and in vivo.

Zhou and colleagues developed an aptamer specific for the HIV-1 gp120 protein expressed on the surface of HIV-infected cells [91, 92]. This anti-gp120 aptamer was fused to a DsiRNA specific for the HIV *tat/rev* gene. The chimeric aptamer–DsiRNA molecule was synthesized by in vitro transcription (IVT) using 2'-F pyrimidine bases to improve nuclease stability, thereby permitting use of the fusion construct in serum without the necessity for additional protection. The aptamer component was shown to target HIV-infected cells and facilitate DsiRNA uptake. Following uptake, the aptamer and DsiRNA are separated by Dicer processing to liberate an active 21-nt siRNA that directs suppression of the HIV *tat/rev* gene. Neff and colleagues studied use of the gp120-aptamer anti-*tat/rev* DsiRNA fusion molecule to suppress HIV infection in a humanized mouse immune system [83]. The humanized Rag2^{-/-} γ c^{-/-} (RAG-hu) mouse is a rodent model system that allows the study of sustained chronic HIV infection. Over time, infected mice show declining

CD4⁺ T cell numbers similar to what is seen in human HIV infection. The gp120-aptamer anti-*tat/rev* DsiRNA fusion molecule was administered intravenously to the RAG-hu mice at a dose of 0.25 nmol (0.38 mg/kg) given as daily injections for 2 days followed by weekly injections for 4 weeks. Mice were monitored for HIV titers and CD4⁺ T cell counts during therapy and for 3–9 weeks after treatment ended. The anti-gp120 aptamer alone lowered viral titers; however, the DsiRNA fusion construct showed the highest efficacy, reducing HIV titers by several logs and preventing the decline of CD4⁺ T cells. The beneficial effects persisted for several weeks following the last dose. Zhou and colleagues employed the same anti-*tat/rev* DsiRNA in RAG-hu mice with a poly(amidoamine) (PAMAM) dendrimer-based nanoparticle to facilitate delivery [84]. DsiRNAs specific for HIV host factors *CD4* and *TNPO3* were also employed. The DsiRNA/dendrimer particles were administered IV at a dose of 0.25 nmol (DsiRNA) given as daily injections for 2 days followed by weekly injections for 4 weeks. Similar to earlier results using aptamer-based delivery, IV administration of the anti-*tat/rev* DsiRNA in HIV-infected humanized mice resulted in a significant reduction of viral titers. The most promising results were obtained using a cocktail of all three DsiRNAs: an anti-*tat/rev* plus anti-*CD4* plus anti-*TNPO3* DsiRNA mix. The PAMAM dendrimer nanoparticles were shown to accumulate in PBMCs and the liver without evidence for toxicity.

2.4 Future Perspectives

Development of nucleic acid-based drugs to treat human disease is an area receiving considerable attention today. RNAi-based therapeutics are currently under development by a number of biotechnology and pharmaceutical companies. Recent reviews by Davidson [93] and Burnett [94] provide a comprehensive overview of human clinical trials that have been completed or are in progress as of mid-2011. Dicerna Pharmaceuticals (Watertown, MA, USA) is specifically focusing on use of DsiRNA as a platform technology for drug development. Working in partnership with the Japanese pharmaceutical company Kyowa Hakko Kirin, oncology and immunological/inflammatory diseases are current areas of focus. DsiRNAs have not yet been used in humans; however, DsiRNA drug development is proceeding forward, and it is hoped that some of the lead compounds will enter clinical trials in the not too distant future.

The DsiRNAs that move forward as therapeutic candidates will likely be more highly modified than the compounds described in this chapter. In particular, more extensive use of nuclease stabilizing groups such as 2'-OMe RNA will be employed with modification of both the sense and antisense strands while still leaving an unmodified Dicer cleavage site available (Fig. 2.5). Increasing the degree of modification will improve half-life of the compounds and reduce risk of immune stimulation. Given the known effects that sequence context has on which precise

modification patterns are tolerated, it is likely that individual modification patterns will be optimized for each clinical candidate rather than using a simple “one size fits all” universal modification approach.

It is possible to suppress the expression of any desired gene using existing RNAi methods *in vitro*. The single greatest impediment to widespread adoption of *in vivo* use of these reagents for both research and medical applications is the availability of effective delivery tools capable of carrying a highly charged anionic RNA duplex across the cell membrane and deliver it intact in an active form to the cytoplasm with low toxicity. A wide variety of delivery tools are under development which may enable use of dsRNAs as drugs. These methods and chemical compositions have been the subject of numerous excellent reviews in recent years, and the reader is referred to these sources for additional details [88, 95–105]. There is no reason to believe that 21-nt siRNAs and 27-nt DsiRNAs will show any difference in their relative efficiency of delivery using cationic lipid or polyplex nanoparticles, so the same tools should be readily applied across both platforms. The DsiRNA platform, however, may offer some advantages when used with some other delivery technologies. For example, it is possible to covalently conjugate carrier molecules to DsiRNAs so that the modifier is removed by Dicer processing and does not remain attached to the final mature 21-nt siRNA that actually enters RISC (e.g., at the 3'-end of the sense strand, see Fig. 2.5). This may offer an advantage for using DsiRNAs when employing delivery tools such as cell-penetrating peptides, aptamers, or other high molecular weight ligands which might interfere with RISC entry. Indeed, pilot *in vivo* studies discussed above in Sect. 2.3 demonstrated the successful use of DsiRNAs covalently conjugated with both large aptamer and CpG-motif oligonucleotides to facilitate delivery.

Synthesis of siRNA and DsiRNA duplexes is available from a variety of commercial sources from very small scale to multi-gram scales to support all research and preclinical needs. A smaller number of suppliers are certified to produce the cGMP quality synthetic oligonucleotides needed for pharmaceutical use in humans. Currently, most cGMP manufacturers employ commercially available synthesis platforms, such as the GE Healthcare OligoPilot™ and OligoProcess™ synthesizers, or the Asahi Kasei TechniKrom® platform, which are capable of doing syntheses in millimole to mole scales, resulting in greater than kilogram yields of final product. Methods to produce cGMP quality synthetic oligonucleotides are well established, largely thanks to the many years of experience gained from clinical trials done using single-stranded antisense oligonucleotides [106, 107].

Acknowledgments The authors thank Kim Lennox for critical reading of the manuscript and Dr. Joe Dobosy for assistance with the figures. We further thank Dr. John Rossi, Dr. Dongho Kim, and all members of the research laboratories at Integrated DNA Technologies for their contributions towards the development of the Dicer-substrate siRNA technology.

Conflict of Interest Statement MAB and SDR are employed by Integrated DNA Technologies (IDT), which sells compounds similar to those described herein. IDT is, however, not a publicly traded company, and the authors do not hold any stock or equity in IDT. MAB is a scientific cofounder of Dicerna Pharmaceuticals and is a member of their Scientific Advisory Board.

References

1. Mello CC, Conte D Jr (2004) Revealing the world of RNA interference. *Nature* 431(7006):338–342
2. Siomi H, Siomi MC (2009) On the road to reading the RNA-interference code. *Nature* 457(7228):396–404
3. Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell* 136(4):642–655
4. Ambros V (2004) The functions of animal microRNAs. *Nature* 431(7006):350–355
5. Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature* 431(7006):343–349
6. Vickers TA, Lima WF, Wu H, Nichols JG, Linsley PS, Crooke ST (2009) Off-target and a portion of target-specific siRNA mediated mRNA degradation is Ago2 ‘Slicer’ independent and can be mediated by Ago1. *Nucleic Acids Res* 37(20):6927–6941
7. Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409(6818):363–366
8. Zhang H, Kolb FA, Brondani V, Billy E, Filipowicz W (2002) Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *EMBO J* 21(21):5875–5885
9. Okamura K, Lai EC (2008) Endogenous small interfering RNAs in animals. *Nat Rev Mol Cell Biol* 9(9):673–678
10. Liu Q, Rand TA, Kalidas S, Du F, Kim HE, Smith DP et al (2003) R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. *Science* 301(5641):1921–1925
11. Haase AD, Jaskiewicz L, Zhang H, Laine S, Sack R, Gagnon A et al (2005) TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. *EMBO Rep* 6(10):961–967
12. Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K et al (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* 436(7051):740–744
13. MacRae IJ, Zhou K, Li F, Repic A, Brooks AN, Cande WZ et al (2006) Structural basis for double-stranded RNA processing by Dicer. *Science* 311(5758):195–198
14. MacRae IJ, Zhou K, Doudna JA (2007) Structural determinants of RNA recognition and cleavage by Dicer. *Nat Struct Mol Biol* 14(10):934–940
15. Maniatakis E, Mourelatos Z (2005) A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. *Genes Dev* 19(24):2979–2990
16. Sontheimer EJ (2005) Assembly and function of RNA silencing complexes. *Nat Rev Mol Cell Biol* 6(2):127–138
17. Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* 123(4):607–620
18. Rand TA, Petersen S, Du F, Wang X (2005) Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* 123(4):621–629
19. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R (2005) Human RISC couples MicroRNA biogenesis and posttranscriptional gene silencing. *Cell* 123(4):631–640
20. Lingel A, Simon B, Izaurralde E, Sattler M (2004) Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. *Nat Struct Mol Biol* 11(6):576–577
21. Okamura K, Ishizuka A, Siomi H, Siomi MC (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev* 18(14):1655–1666
22. Peters L, Meister G (2007) Argonaute proteins: mediators of RNA silencing. *Mol Cell* 26(5):611–623
23. Hutvagner G, Simard MJ (2008) Argonaute proteins: key players in RNA silencing. *Nat Rev Mol Cell Biol* 9(1):22–32

24. Wang B, Li S, Qi HH, Chowdhury D, Shi Y, Novina CD (2009) Distinct passenger strand and mRNA cleavage activities of human Argonaute proteins. *Nat Struct Mol Biol* 16(12):1259–1266
25. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* 15(2):185–197
26. Ameres SL, Martinez J, Schroeder R (2007) Molecular basis for target RNA recognition and cleavage by human RISC. *Cell* 130(1):101–112
27. Wang HW, Noland C, Siridechadilok B, Taylor DW, Ma E, Felderer K et al (2009) Structural insights into RNA processing by the human RISC-loading complex. *Nat Struct Mol Biol* 16(11):1148–1153
28. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411(6836):494–498
29. Chang CI, Kim HA, Dua P, Kim S, Li CJ, Lee DK (2011) Structural diversity repertoire of gene silencing small interfering RNAs. *Nucleic Acid Ther* 21(3):125–131
30. Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ (2005) Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol* 23(2):222–226
31. Rose SD, Kim DH, Amarzguioui M, Heidel JD, Collingwood MA, Davis ME et al (2005) Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic Acids Res* 33(13):4140–4156
32. Sano M, Sierant M, Miyagishi M, Nakanishi M, Takagi Y, Sutou S (2008) Effect of asymmetric terminal structures of short RNA duplexes on the RNA interference activity and strand selection. *Nucleic Acids Res* 36(18):5812–5821
33. Aza-Blanc P, Cooper CL, Wagner K, Batalov S, Deveraux QL, Cooke MP (2003) Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. *Mol Cell* 12(3):627–637
34. Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell* 115(2):209–216
35. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115(2):199–208
36. Noland CL, Ma E, Doudna JA (2011) siRNA repositioning for guide strand selection by human Dicer complexes. *Mol Cell* 43(1):110–121
37. Hefner E, Clark K, Whitman C, Behlke MA, Rose SD, Peek AS et al (2008) Increased potency and longevity of gene silencing using validated Dicer substrates. *J Biomol Tech* 19(4):231–237
38. Eder PS, DeVine RJ, Dagle JM, Walder JA (1991) Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3' exonuclease in plasma. *Antisense Res Dev* 1(2):141–151
39. Kurreck J (2003) Antisense technologies Improvement through novel chemical modifications. *Eur J Biochem* 270(8):1628–1644
40. Behlke MA (2008) Chemical modification of siRNAs for in vivo use. *Oligonucleotides* 18(4):305–320
41. Gaglione M, Messere A (2010) Recent progress in chemically modified siRNAs. *Mini Rev Med Chem* 10(7):578–595
42. Krieg AM, Stein CA (1995) Phosphorothioate oligodeoxynucleotides: antisense or anti-protein? *Antisense Res Dev* 5(4):241
43. Braasch DA, Jensen S, Liu Y, Kaur K, Arar K, White MA et al (2003) RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry* 42(26):7967–7975
44. Amarzguioui M, Holen T, Babaie E, Prydz H (2003) Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res* 31(2):589–595
45. Chiu YL, Rana TM (2003) siRNA function in RNAi: a chemical modification analysis. *RNA* 9(9):1034–1048

46. Czauderna F, Fechtner M, Dames S, Aygun H, Klippel A, Pronk GJ et al (2003) Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Res* 31(11):2705–2716
47. Harborth J, Elbashir SM, Vandenberg K, Manninga H, Scaringe SA, Weber K et al (2003) Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev* 13(2):83–105
48. Choung S, Kim YJ, Kim S, Park HO, Choi YC (2006) Chemical modification of siRNAs to improve serum stability without loss of efficacy. *Biochem Biophys Res Commun* 342(3):919–927
49. Allerson CR, Sioufi N, Jarres R, Prakash TP, Naik N, Berdeja A et al (2005) Fully 2'-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. *J Med Chem* 48(4):901–904
50. Prakash TP, Allerson CR, Dande P, Vickers TA, Sioufi N, Jarres R et al (2005) Positional effect of chemical modifications on short interference RNA activity in mammalian cells. *J Med Chem* 48(13):4247–4253
51. Kraynack BA, Baker BF (2006) Small interfering RNAs containing full 2'-O-methylribonucleotide-modified sense strands display Argonaute2/eIF2C2-dependent activity. *RNA* 12(1):163–176
52. Layzer JM, McCaffrey AP, Tanner AK, Huang Z, Kay MA, Sullenger BA (2004) In vivo activity of nuclease-resistant siRNAs. *RNA* 10(5):766–771
53. Morrissey DV, Blanchard K, Shaw L, Jensen K, Lockridge JA, Dickinson B et al (2005) Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. *Hepatology* 41(6):1349–1356
54. Morrissey DV, Lockridge JA, Shaw L, Blanchard K, Jensen K, Breen W et al (2005) Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat Biotechnol* 23(8):1002–1007
55. Swayze EE, Siwkowski AM, Wancewicz EV, Migawa MT, Wyrzykiewicz TK, Hung G et al (2007) Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals. *Nucleic Acids Res* 35(2):687–700
56. Elmen J, Thonberg H, Ljungberg K, Frieden M, Westergaard M, Xu Y et al (2005) Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res* 33(1):439–447
57. Elmen J, Lindow M, Silahtaroglu A, Bak M, Christensen M, Lind-Thomsen A et al (2008) Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic Acids Res* 36(4):1153–1162
58. Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S et al (2008) LNA-mediated microRNA silencing in non-human primates. *Nature* 452(7189):896–899
59. Kubo T, Zhelev Z, Ohba H, Bakalova R (2007) Modified 27-nt dsRNAs with dramatically enhanced stability in serum and long-term RNAi activity. *Oligonucleotides* 17(4):445–464
60. Kubo T, Zhelev Z, Ohba H, Bakalova R (2008) Chemically modified symmetric and asymmetric duplex RNAs: an enhanced stability to nuclease degradation and gene silencing effect. *Biochem Biophys Res Commun* 365(1):54–61
61. Turner JJ, Jones SW, Moschos SA, Lindsay MA, Gait MJ (2007) MALDI-TOF mass spectral analysis of siRNA degradation in serum confirms an RNase A-like activity. *Mol Biosyst* 3(1):43–50
62. Collingwood MA, Rose SD, Huang L, Hillier C, Amarzguioui M, Wiiger MT et al (2008) Chemical modification patterns compatible with high potency dicer-substrate small interfering RNAs. *Oligonucleotides* 18(2):187–200
63. Nishina K, Unno T, Uno Y, Kubodera T, Kanouchi T, Mizusawa H et al (2008) Efficient in vivo delivery of siRNA to the liver by conjugation of alpha-tocopherol. *Mol Ther* 16(4):734–740

64. Rigotti A (2007) Absorption, transport, and tissue delivery of vitamin E. *Mol Aspects Med* 28(5–6):423–436
65. Kubo T, Takei Y, Mihara K, Yanagihara K, Seyama T (2012) Amino-modified and lipid-conjugated dicer-substrate siRNA enhances RNAi efficacy. *Bioconjug Chem* 23(2):164–173
66. Kariko K, Buckstein M, Ni H, Weissman D (2005) Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* 23(2):165–175
67. Robbins M, Judge A, Liang L, McClintock K, Yaworski E, MacLachlan I (2007) 2'-O-methyl-modified RNAs Act as TLR7 Antagonists. *Mol Ther* 15(9):1663–1669
68. Gantier MP, Williams BR (2007) The response of mammalian cells to double-stranded RNA. *Cytokine Growth Factor Rev* 18(5–6):363–371
69. Robbins M, Judge A, MacLachlan I (2009) siRNA and innate immunity. *Oligonucleotides* 19(2):89–102
70. Reynolds A, Anderson EM, Vermeulen A, Fedorov Y, Robinson K, Leake D et al (2006) Induction of the interferon response by siRNA is cell type- and duplex length-dependent. *RNA* 12(6):988–993
71. Fedorov Y, King A, Anderson E, Karpilow J, Ilsley D, Marshall W et al (2005) Different delivery methods-different expression profiles. *Nat Methods* 2(4):241
72. Marques JT, Devosse T, Wang D, Zamanian-Daryoush M, Serbinowski P, Hartmann R et al (2006) A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nat Biotechnol* 24(5):559–565
73. Behlke MA (2006) Progress towards in vivo use of siRNAs. *Mol Ther* 13(4):644–670
74. Rettig GR, Behlke MA (2012) Progress towards in vivo use of siRNAs-II. *Mol Ther* 20:483–512
75. Lundberg P, Welander PV, Edwards CK 3rd, van Rooijen N, Cantin E (2007) Tumor necrosis factor (TNF) protects resistant C57BL/6 mice against herpes simplex virus-induced encephalitis independently of signaling via TNF receptor 1 or 2. *J Virol* 81(3):1451–1460
76. Lundberg P, Yang H-J, Jung S-J, Behlke MA, Rose SD, Cantin EM (2012) Protection against TNF α -dependent liver toxicity by intraperitoneal liposome delivered DsiRNA targeting TNF α in vivo. *J Control Release* 160:194–199
77. Howard KA, Paludan SR, Behlke MA, Besenbacher F, Deleuran B, Kjems J (2008) Chitosan/siRNA nanoparticle-mediated TNF-alpha knockdown in peritoneal macrophages for anti-inflammatory treatment in a murine arthritis model. *Mol Ther* 17(1):162–168
78. Nawroth I, Alsner J, Behlke MA, Besenbacher F, Overgaard J, Howard KA et al (2010) Intraperitoneal administration of chitosan/DsiRNA nanoparticles targeting TNFalpha prevents radiation-induced fibrosis. *Radiother Oncol* 97(1):143–148
79. Dore-Savard L, Roussy G, Dansereau MA, Collingwood MA, Lennox KA, Rose SD et al (2008) Central delivery of Dicer-substrate siRNA: a direct application for pain research. *Mol Ther* 16(7):1331–1339
80. LaCroix-Fralish ML, Mo G, Smith SB, Sotocinal SG, Ritchie J, Austin JS et al (2009) The beta3 subunit of the Na⁺, K⁺-ATPase mediates variable nociceptive sensitivity in the formalin test. *Pain* 144(3):294–302
81. Sato Y, Murase K, Kato J, Kobune M, Sato T, Kawano Y et al (2008) Resolution of liver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone. *Nat Biotechnol* 26(4):431–442
82. Kortylewski M, Swiderski P, Herrmann A, Wang L, Kowolik C, Kujawski M et al (2009) In vivo delivery of siRNA to immune cells by conjugation to a TLR9 agonist enhances anti-tumor immune responses. *Nat Biotechnol* 27(10):925–932
83. Neff CP, Zhou J, Remling L, Kuruvilla J, Zhang J, Li H et al (2011) An aptamer-siRNA chimera suppresses HIV-1 viral loads and protects from helper CD4(+) T cell decline in humanized mice. *Sci Transl Med* 3(66):66ra6
84. Zhou J, Neff CP, Liu X, Zhang J, Li H, Smith DD et al (2011) Systemic administration of combinatorial dsRNAs via nanoparticles efficiently suppresses HIV-1 infection in humanized mice. *Mol Ther* 19(12):2228–2238

85. Amarzguoui M, Lundberg P, Cantin E, Hagstrom JE, Behlke MA, Rossi JJ (2006) Rational design and in vitro and in vivo delivery of Dicer substrate siRNA. *Nat Protoc* 1(2):508–517
86. Howard KA, Rahbek UL, Liu X, Damgaard CK, Glud SZ, Andersen MO et al (2006) RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticle system. *Mol Ther* 14(4):476–484
87. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R et al (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374(6522):546–549
88. Thiel KW, Giangrande PH (2009) Therapeutic applications of DNA and RNA aptamers. *Oligonucleotides* 19(3):209–222
89. Zhou J, Rossi JJ (2010) Aptamer-targeted cell-specific RNA interference. *Silence* 1(1):4
90. Syed MA, Pervaiz S (2010) Advances in aptamers. *Oligonucleotides* 20(5):215–224
91. Zhou J, Li H, Li S, Zaia J, Rossi JJ (2008) Novel dual inhibitory function aptamer-siRNA delivery system for HIV-1 therapy. *Mol Ther* 16(8):1481–1489
92. Zhou J, Swiderski P, Li H, Zhang J, Neff CP, Akkina R et al (2009) Selection, characterization and application of new RNA HIV gp 120 aptamers for facile delivery of Dicer substrate siRNAs into HIV infected cells. *Nucleic Acids Res* 37(9):3094–3109
93. Davidson BL, McCray PB Jr (2011) Current prospects for RNA interference-based therapies. *Nat Rev Genet* 12(5):329–340
94. Burnett JC, Rossi JJ, Tiemann K (2011) Current progress of siRNA/shRNA therapeutics in clinical trials. *Biotechnol J* 6(9):1130–1146
95. De Paula D, Bentley MV, Mahato RI (2007) Hydrophobization and bioconjugation for enhanced siRNA delivery and targeting. *RNA* 13(4):431–456
96. de Fougères AR (2008) Delivery vehicles for small interfering RNA in vivo. *Hum Gene Ther* 19(2):125–132
97. Howard KA, Kjems J (2007) Polycation-based nanoparticle delivery for improved RNA interference therapeutics. *Expert Opin Biol Ther* 7(12):1811–1822
98. Howard KA (2009) Delivery of RNA interference therapeutics using polycation-based nanoparticles. *Adv Drug Deliv Rev* 61(9):710–720
99. Tseng YC, Mozumdar S, Huang L (2009) Lipid-based systemic delivery of siRNA. *Adv Drug Deliv Rev* 61(9):721–731
100. Davis ME (2009) The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic. *Mol Pharm* 6(3):659–668
101. Eguchi A, Dowdy SF (2009) siRNA delivery using peptide transduction domains. *Trends Pharmacol Sci* 30(7):341–345
102. Jarver P, Mager I, Langel U (2010) In vivo biodistribution and efficacy of peptide mediated delivery. *Trends Pharmacol Sci* 31(11):528–535
103. Giljohann DA, Seferos DS, Daniel WL, Massich MD, Patel PC, Mirkin CA (2010) Gold nanoparticles for biology and medicine. *Angew Chem Int Ed Engl* 49(19):3280–3294
104. Serda RE, Godin B, Blanco E, Chiappini C, Ferrari M (2011) Multi-stage delivery nanoparticle systems for therapeutic applications. *Biochim Biophys Acta* 1810(3):317–329, Epub 2010/05/25
105. Peer D, Lieberman J (2011) Special delivery: targeted therapy with small RNAs. *Gene Ther* 18(12):1127–1133
106. Sanghvi YS, Schulte M (2004) Therapeutic oligonucleotides: the state-of-the-art in purification technologies. *Curr Opin Drug Discov Devel* 7(6):765–776
107. Tedebark U, Scozzari A, Werbitzky O, Capaldi D, Holmberg L (2011) Industrial-scale manufacturing of a possible oligonucleotide cargo CPP-based drug. *Methods Mol Biol* 683:505–524, Epub 2010/11/06



<http://www.springer.com/978-1-4614-4744-3>

RNA Interference from Biology to Therapeutics

Howard, K.A. (Ed.)

2013, XVI, 340 p.,

ISBN: 978-1-4614-4744-3