INTEGRATED DNA TECHNOLOGIES

RNA Interference (RNAi) and DsiRNAs

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1. Introduction to RNAi

RNA interference is a conserved pathway found in most eukaryotes where double-stranded RNAs (dsRNAs) suppress expression of genes with complementary sequences [1, 2]. dsRNAs were discovered to serve as effective triggers of RNAi in the nematode, *Caenorhabditis elegans* [4]. Since then, RNAi has become the experimental tool of choice for studying the effects of gene silencing.

Long dsRNAs are degraded by the endoribonuclease Dicer into small effector molecules called siRNAs (small interfering RNAs). SiRNAs are usually around 21 base pairs (bp) long with a central 19 bp duplex and 2-base 3'-overhangs. In mammals, Dicer processing occurs in a multiprotein complex with the RNA-binding protein TRBP. The nascent siRNA associates with Dicer, TRBP, and Argonaute 2 (Ago2) to form the RNA-Induced Silencing Complex, or RISC [5]. Once in RISC, one strand of the siRNA (the passenger strand) is degraded or discarded while the other strand (the guide strand) remains to direct sequence specificity of the silencing complex. The Ago2 component of RISC is a ribonuclease that will cleave the target RNA under direction of the guide strand. A schematic overview of the pathways involved in degradative RNAi is shown in Figure 1. Once the RISC complex is activated, it can move on to target additional mRNA targets. This effect amplifies gene silencing and allows the therapeutic effect to last for 3-7 days in rapidly dividing cells or several weeks in non-dividing cells [6].

Many researchers today employ synthetic RNA duplexes as their RNAi reagents, which mimic the natural siRNAs that result from Dicer processing of long substrate RNAs. These synthetic

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RNA duplexes are transfected into cell lines where they mimic *in vivo* Dicer products. Although long (several hundred bp) dsRNAs are commonly employed to trigger RNAi in *C. elegans* or *D. melanogaster*, these molecules will activate the innate immune system to trigger interferon (IFN) responses in higher organisms. RNAi can be performed in mammalian cells using short RNAs, which generally do not induce IFN responses.

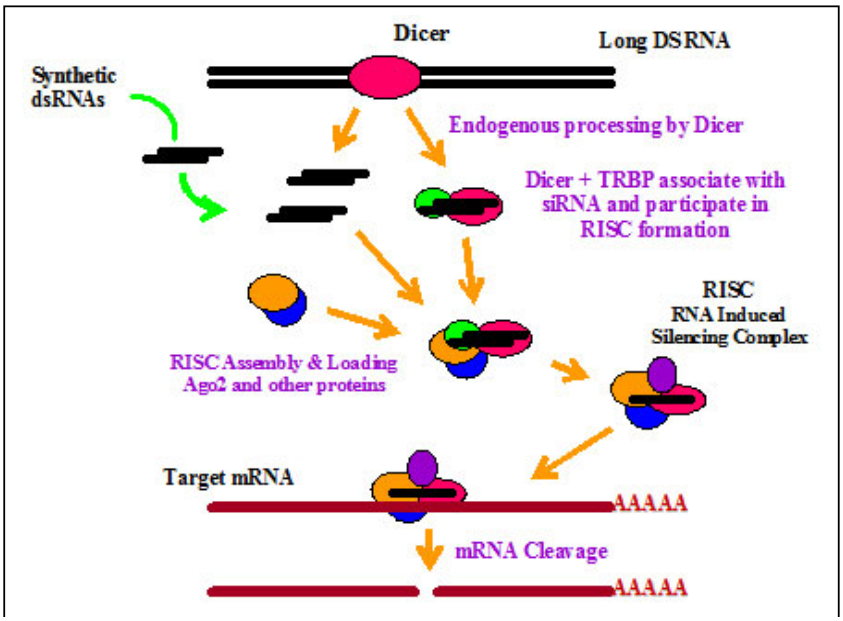

Figure 1. Pathways involved in degradative RNAi. Double-stranded RNAs (DsiRNAs) are cleaved by Dicer into small interfering RNAs (siRNAs) that are 21- to 23-base pair duplexes with 3' overhangs and 5'-phosphates. The siRNAs then enter the RNA-induced silencing complex (RISC) where the guide strand directs cleavage of the target RNA. [3]

2. Dicer-Substrate RNAi Technology

Traditionally, siRNAs are chemically synthesized as 21-mers with a central 19 bp duplex region and symmetric 2-base 3' overhangs on each end. Dicer-substrate RNAs (DsiRNAs) are chemically synthesized 27-mer RNA duplexes that were developed as a collaborative effort between John Rossi (Beckman Research Institute of the City of Hope) and IDT. DsiRNAs have an increased potency in RNA interference of up to 100-fold when compared to conventional 21-mer siRNAs [7]. In addition, DsiRNAs are able to effectively target some sites that 21-mers are not able to silence [7].

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DsiRNAs are processed by Dicer into 21-mer siRNAs and designed so that cleavage results in a single, desired product. This is possible due to a novel asymmetric design where the RNA duplex has a single 2-base 3'-overhang on the antisense strand and is blunt on the other end; the blunt end is modified with DNA bases, shown in Figure 2. This design provides Dicer with a single favorable PAZ binding site that helps direct the cleavage reaction. Functional polarity is introduced by this processing event, which favors antisense strand loading into RISC; the increased potency of these reagents is thought to relate to linkage between Dicer processing and RISC loading [8]. Increased antisense loading will result in increased sense mRNA cleavage.

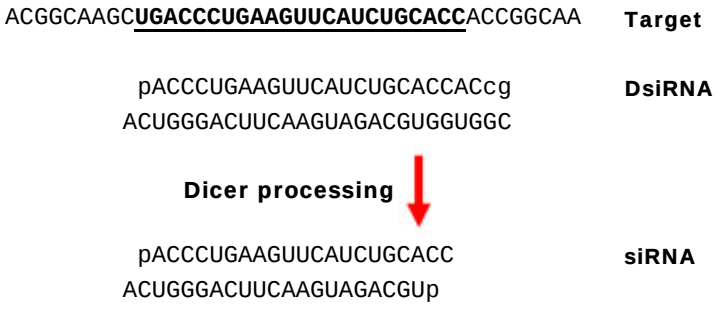

Figure 2. Design of a DsiRNA. The DsiRNA has a single 2-base 3'-overhang on the antisense strand and a blunt end modified with DNA bases (shown in lower case). This is processed by Dicer into an siRNA with symmetric 2-base 3' overhangs on each end.

3. Designing siRNAs

The ability of an siRNA to silence gene expression is predominantly determined by its sequence and not all target sites are equal [3, 9]. In addition to the actual sequence, other considerations such as cross-hybridization and chemical modifications can alter the effectiveness of the siRNA [3].

3.1 Location

The location of an siRNA within the entire target gene is less of a concern for potency than the localization of the siRNA within a particular gene exon structure [3]. Therefore, knowledge of when specific splice variants are used is important to determine how to most effectively target the desired isoform(s) of the gene.

3.2 Modification

siRNAs must have phosphate groups at the 5' end in order to have activity so it is important to not block the 5' end of the antisense strand with any modifications other than phosphate [3]. However, transfected, unmodified 5'-OH ends are rapidly phosphorylated by cellular kinases which means it is also not necessary to phosphorylate synthetic siRNAs [3]. Regardless of the target sequence, DNA 'TT' dinucleotide overhangs are often added to the 3' ends; however some evidence suggests that using RNA bases may lead to greater potency [3].

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3.3 Thermodynamic stability

The thermodynamic properties of siRNAs have a significant influence on their potency; thermodynamic asymmetry is fundamentally important for siRNA function and strand loading into RISC [3]. The most effective siRNAs have a relatively low T_m and duplex stability (less stable, more A/U rich) toward the 5'-end of the guide strand and a relatively high T_m (more stable, more G/C rich) toward the 5'-end of passenger strand [3]. When options are limited for a particular target sequence, it may not be possible to select thermodynamically favorable regions. For these situations, it is possible to introduce mismatches (to lower T_m) or to add modified bases (to increase T_m) to the siRNA duplex to create thermodynamic asymmetry [3]. If non-complementary bases must be introduced, it is important that it is at the 3'-end of the sense strand (passenger strand) rather than the 5'-end of the AS strand (guide strand) to avoid impairing the ability of the AS strand to anneal to the active target [3].

3.4 Sequence characteristics and specificity

To maintain specificity, certain sequence characteristics should be avoided in the guide strand such as homopolymeric runs (those with four or more identical nucleotides) and nine-base or greater segments of G/C bases [3]. In addition, the secondary structure of the target and the site accessibility are important factors in the activity of siRNAs [3]. A moderate to low GC content (30 to 52%) tends to be a feature of functional siRNAs [3].

Due to the high specificity of nucleic acid base pairing, even a single mismatch in a 19-base sequence can prevent duplex formation; however, cross hybridization can and does occur [3]. For this reason, it is very important to screen all candidate siRNAs for homology to other targets and exclude those with significant complementarity [3]. BLAST is not a good tool for finding short five- to eight-base domains of sequence identity that may exist between a candidate siRNA and other genes; the Smith-Waterman algorithm is recommended for siRNA homology screening instead [3]. Programs such as SSEARCH or JALIGNER are two free options for this type of analysis.

Like targeted effects, off-target effects (OTEs) are dose dependent. Therefore, it is important to establish dose-response profiles for all siRNAs in use and always use the lowest concentration of siRNA that will provide adequate target knockdown. An additional measure to prevent OTE bias is to ensure that at least two, and ideally three, independent siRNAs against a target give the same result [3].

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4. Chemical Modifications

While chemical modification of RNAi oligos is not required for siRNA function, certain modifications are sometimes useful. Chemical modifications can decrease the susceptibility of synthetic nucleic acids to nuclease degradation and reduce their ability to trigger an innate immune response during *in vivo* applications [10]. In addition, chemical modification can be used to prevent unwanted participation in miRNA pathways that create off-target effects as

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well as help with cellular uptake [10]. However, chemical modification can also alter the potency of a siRNA. Some modification patterns can have a greater impact on potency than others; therefore, modified siRNAs must be empirically tested to ensure they are effective.

In order to be functional, siRNA must be able to interact with cellular proteins. This interaction might be affected by changes to the RNA structure caused by modifications. The easiest way to increase nuclease stability is to modify the internucleotide phosphate linkage rather than to attach additional groups to the bases [10]. A non-bridging oxygen can be replaced with sulfur (phosphorothioate), boron (boranophosphate), nitrogen (phosphoramidate), or methyl (methylphosphonate) groups to provide nuclease resistance and stabilization [10]. The phosphorothioate modification is available at IDT.

The antisense strand must have either a free 5'-OH or 5' phosphate terminus [11, 12]. As stated above, siRNAs must have phosphate groups at the 5' end in order to have activity so it is important to not block the 5' end of the antisense strand with any modifications other than phosphate [3]. However, transfected, unmodified 5'-OH ends are rapidly phosphorylated by cellular kinases which means it is also not necessary to phosphorylate synthetic siRNAs [3]. A 5' phosphate results in natural Dicer processing and is the active form of the molecule. For 21-mer siRNAs, 5'-end modification of the sense strand RNA does not alter the efficacy of silencing and addition of a fluorescent dye, biotin, or other similar modifier can safely be done at this position. In the traditional 21-mer siRNA design, the single-stranded 3' are particularly susceptible to degradation by exonucleases. These overhangs can be protected from degradation with an inverted-dT base or other non-nucleotide groups at this position [10]. In contrast, longer RNAs, such as 27-mer Dicer-substrate duplexes, appear to have greater inherent protection against nucleases [10].

Chemical modification of DsiRNAs is slightly different due to the need to interact with Dicer. Modifications placed at the 3'-end of the sense strand have minimal impact on function or potency. Therefore, IDT recommends this position for routine processing. If a modification (such as biotin) is desired to be retained in the siRNA after cleavage, then 5'-S modification is preferred.

4.1 Nuclease resistance and stability

All RNAs have the potential to trigger IFN responses in cells [13, 14]. Certain sequences and cell types are more at risk. Incorporation of 2'-O-methyl RNA residues can prevent activation of IFN responses [15] and should be considered for all *in vivo* applications. Extensive modification with 2'OMe RNA can reduce potency or completely inactivate the siRNA. Instead, we recommend alternating 2'OMe with unmodified RNA bases which will generally retain siRNA function and also provide significant nuclease stabilization [10]. Most 2'-modifications are not naturally occurring and so may confer toxic side effects. The 2'OMe modification, in contrast, is a naturally occurring RNA variant and is not thought to create significant toxicity when used in synthetic siRNAs [10].

A number of other modifications can also be used to provide nuclease resistance and stability:

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- Modifying the 2'-position of the ribose may indirectly improve the nuclease resistance of the internucleotide phosphate bond as well as increase the duplex stability and provide protection from immune activation [10].
- Modification with 2'-fluoro (2'-F) maintains siRNA function and also helps stabilize the duplex against nuclease degradation [10].
- Both stability and improved *in vivo* performance can be achieved by combining 2'-F pyrimidines with 2'OMe purines [10].
- Limited incorporation of LNA bases can provide significant nuclease stabilization. However, extensive modification with LNA bases generally results in decreased activity to a greater degree than 2'OMe modification [10].
- Substitution of DNA bases into a siRNA may offer advantages including stabilization against ribonucleases [10].
- Attachment of steroid and other hydrophobic lipid groups can alter protein binding in serum, extend circulation time, and facilitate direct cellular uptake [10].
- IDT recommends use of TYE™563 or Cy3™ when a fluorescent RNA is desired. These dyes are brighter than fluorescein-based dyes and do not fall within the wavelengths where cellular autofluorescence commonly occurs.

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5. *In vivo* use of siRNAs

Use of siRNAs *in vivo* shows great potential as both research tools and as therapeutic agents [16]. Delivering the siRNA to the target is the most significant barrier to the widespread use of siRNA therapeutics [6]. Before beginning RNAi studies *in vivo,* the following issues should be considered: site selection, compound design and chemistry, controls, route of administration, and use of a delivery vehicle [16]. It is very important to validate siRNA duplexes *in vitro* before moving into *in vivo* experiments in order to find the best candidates. In addition, it is best to find more than one effective siRNA for each target that will be tested in order to control for off-target effects [16]. DsiRNAs have been successfully used *in vivo* [9] and are available from milligram to gram scale both as chemically stabilized and as unmodified RNA.

siRNAs can be rapidly degraded inside cells if they are not protected from nuclease attack [10]. Protection can be in the form of a delivery tool or by adding nuclease resistant modifications to the siRNA [10]. IDT recommends 2' OMe modifications for siRNAs that will be used *in vivo*. RNA synthesized by *in vitro* transcription can trigger an IFN response due to the retention of a triphosphate group at the 5' end. Using a chemically synthesized RNA can avoid this issue and also provide known structure and purity [16].

In order to ensure that unsuspected OTEs are not creating misleading results, it is important to have two different siRNAs against the same target produce the same results [16]. It may be impossible to ensure that any single negative control siRNA is not causing OTEs as well. Measuring serum cytokine levels can provide information on whether or not IFN induction has

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occurred [16]. Gene knockdown as well as stimulation of IFN pathways and OTEs are all dose dependent. It is important to establish dose-response profiles that are specific for the *in vivo* application for all siRNAs in use and always use the lowest concentration of siRNA that will provide adequate target knockdown or therapeutic effect.

The route in which the siRNA is administered will affect many aspects of the experiments including: the total dose needed, the effective tissue distribution, potential side effects, and the need for chemical modification or use of an agent that facilitates delivery [16].

Delivery methods include:

- Local delivery through direct injection or topical application to a surface [16]
    - Localized delivery of siRNAs provides the benefit of higher bioavailability to the target tissue and reduced adverse effects that typically go along with systemic administration [6].
    - Several tissues can be treated topically or locally including the eye, skin, mucus membranes, and local tumors [6]. Lung diseases and infections are also particularly well-suited for local siRNA delivery [6].
- Intravenous (iv) injection [16]
- Intraperitoneal (ip) injection [16]
- Subcutaneous (sc) injection [16]
- Intrathecal or intraventricular injection for direct administration into the central nervous system (CNS) [16]
- Inhaled, intranasal, or intratracheal administration for pulmonary delivery [16]
    - This route allows the siRNA to directly contact the lung epithelial cells [6]
- *In vivo* experiments can also be achieved by using *ex vivo* delivery methods. For example, cells can be transfected with siRNAs using standard tissue culture methods and then implanted into a recipient animal [16].
- A wide variety of reagents can be used to facilitate *in vivo* delivery of nucleic acids [16]. Delivery reagents provide facilitated uptake, protection for the siRNA, and help prevent nonspecific delivery [6].
    - Cationic lipids
    - Polyethylenimine (PEI)
    - Other polycations
    - Modified virus envelopes
    - Physical and mechanical delivery methods

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6. Summary

RNAi is a powerful tool for studying gene silencing and its effects. Here we have described important design parameters, the importance of considering off-target effects, and means for achieving *in vivo* gene silencing. Advancements to the technology, such as IDT's DsiRNAs, have led to even greater advancements in the potency of RNA interference. These tools will continue

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to provide the means to study the role specific genes play and the effects of silencing them. For more information on DsiRNAs from IDT, visit the Custom DsiRNA product page at www.idtdna.com.

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