

On the art of identifying effective and specific siRNAs

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Small interfering RNAs (siRNAs) have been widely exploited for sequence-specific gene knockdown, predominantly to investigate gene function in cultured vertebrate cells, and also hold promise as therapeutic agents. Because not all siRNAs that are cognate to a given target mRNA are equally effective, computational tools have been developed based on experimental data to increase the likelihood of selecting effective siRNAs. Furthermore, because target-complementary siRNAs can also target other mRNAs containing sequence segments that are partially complementary to the siRNA, most computational tools include ways to reduce potential off-target effects in the siRNA selection process. Though these methods facilitate selection of functional siRNAs, they do not yet alleviate the need for experimental validation. This perspective provides a practical guide based on current wisdom for selecting siRNAs.

The evolutionarily conserved processes whereby small double-stranded (ds)RNAs of distinct size and structure sequence-specifically suppress the expression of their target genes are referred to as RNA silencing or RNA interference (RNAi)¹. Among the repertoire of known small RNAs, siRNAs mediate gene-specific silencing primarily via recognizing and inducing degradation of the mRNAs of targeted genes. Consequently, siRNAs have become one of the most valuable reagents to functionally annotate genomes and possess great potential as therapeutics^{2–4}.

Shortly after the discovery that siRNA duplexes can specifically silence mammalian genes, it was thought that almost any target-complementary siRNA effectively and specifically silences its cognate target gene⁵. In practice, however, different siRNAs often manifest a spectrum of potency, and only a fraction of them are highly effective⁶. Small positional shifts along the target mRNA were sufficient to alter siRNA function in an apparently unpredictable manner^{6–8}. Moreover, siRNAs may nonspecifically target unrelated genes with only partial sequence-complementarity (off-target effects)^{9–13}. Hence, it is critical to identify effective and specific siRNA sequences to perform reliable gene-knockdown experiments.

Initially, empirical rules had been proposed for siRNA selection, some of which were based on the first identified functional siRNAs⁵. The evolving understanding of the RNAi mechanism, together with statistical analyses of libraries of siRNAs with experimentally determined efficiency, led to computer-based approaches that increased the likelihood of identifying effective and specific siRNAs^{6,14,15}. These tools, however, are not perfect. (i) Not every selected siRNA meets the desired thresholds of potency and specificity, so that experimental proof of downregulation of targeted mRNA or protein remains important, not even considering the evaluation of potential off-target effects. (ii) A substantial fraction of active siRNAs may be dismissed because the weighing of factors influencing activity is complex and partly undefined^{6,9,16}. Not surprisingly, experimental approaches to generate and identify effective siRNAs have been developed to complement rule-driven siRNA selection strategies^{16–18}.

There are many excellent recent reviews covering the mechanism of RNAi^{19–22}. Elements of this mechanism that are important for the selection of siRNA are summarized in **Box 1**.

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BOX 1 siRNA-MEDIATED GENE SILENCING IN MAMMALIAN CELLS: ELEMENTS OF THE MECHANISM

RNAi is a gene-regulatory mechanism triggered by dsRNAs. siRNAs, which consist of duplexes of 21–23 nt RNAs that are base-paired with 2-nt 3' overhangs, mimic intermediates of the natural processing of longer double-stranded RNA triggers by RNase III. The major products of RNase III processing are microRNAs (miRNAs), which are endogenous ~ 22-nt RNAs that repress gene expression by targeting mRNAs for cleavage or translational repression^{22,72}.

An siRNA is generally designed to be fully complementary to its target mRNA and is commonly a product of chemical synthesis. Though the natural processing intermediates carry 5' phosphates, the 5' phosphate is typically omitted in chemical synthesis as a cellular kinase rapidly phosphorylates the siRNA once it is delivered to the cells⁸⁶. Individual siRNAs or random siRNA pools can also be generated by enzymatic methods from digestion of longer dsRNAs^{65,87,88}. Alternatively, siRNAs can be generated by processing of ectopically expressed short hairpin RNAs^{89–91}.

Naturally occurring siRNAs cognate to cellular or viral mRNAs have not been experimentally detected in mammals. Instead, the mammalian RNAi machinery appears to have been adapted solely for miRNA-mediated regulation of mRNAs containing miRNA

binding sites predominantly located in the 3' UTR⁹². Although the majority of miRNAs affect the stability and translation of mRNAs that are only partially complementary^{72,92}, some miRNAs use, like siRNAs, near-perfect complementarity to cleave their targets⁹³. The latter requirement might explain the evolutionarily conserved catalytic aspect of targeted mRNA degradation in mammalian RNAi.

RNase III and/or other components of the RNAi machinery specifically recognize an siRNA duplex and selectively incorporate one of the siRNA strands into different RISCs, including the catalytic endonuclease-containing complex, which is responsible for the strong siRNA gene-knockdown effect^{19,20}. The strand antisense to the targeted mRNA is often referred to as the guide strand, and its base-paired sense strand is known as the passenger strand, which is destroyed upon incorporation of the guide strand into RISC^{47,94,95} (**Fig. 1**). The catalytic RISC recognizes mRNAs containing perfect or near-perfect complementary sequence to the guide siRNA and cleaves the mRNAs at a site precisely 10 nt upstream of the nucleotide opposite the 5'-most nucleotide of the guide strand (**Fig. 1**). The mRNA fragments are subsequently degraded by cellular nucleases, resulting in knockdown of the expression of the corresponding genes.

Here we provide a practical guide and an overview of the theoretical basis for identification and selection of effective and specific siRNAs.

A GUIDE FOR siRNA SELECTION

Target mRNA analysis

The selection of siRNAs against a gene of interest starts with an annotated target mRNA sequence, including its 5' and 3' untranslated regions (UTRs), splice, polymorphic and allelic variants. Because the coding sequence is the most reliable mRNA sequence information available, it is commonly targeted. The UTRs are generally less well characterized, but can also be targeted with similar gene-knockdown efficiency^{8,23,24}. Though it has often been recommended to avoid targeting sequences that contain known binding sites for mRNA-binding proteins, such as the exon-exon junction complex, there is no detailed experimental study available to assess the importance of this guideline.

For practical reasons, selection of siRNAs is often carried out with additional constraints, for example identifying siRNAs that target (i) orthologs in more than one species or (ii) all possible splice variants of a gene.

Database search for published and validated siRNAs

Several databases archive experimentally tested siRNA sequences from the literature^{25–27}. Additionally, validated siRNAs can be acquired from commercial resources (for example, the *Silencer* validated siRNAs from Ambion and HP validated siRNAs from Qiagen). Some vendors, such as Ambion, Qiagen and Dharmacon also provide predesigned siRNAs or custom siRNA design service. Though prevalidated reagents provide an excellent starting point, the user

still has to examine whether these siRNAs are potent and specific to meet the needs²⁸.

If there are no matches to the target gene of interest in any of these databases or in the literature, it is advisable to select 3–5 candidate siRNAs using available guidelines and tools, and subsequently to validate the reagents.

Selected algorithms and siRNA sequence selection tools

Several siRNA sequence selection algorithms have been developed in recent years that rely on intrinsic sequence and stability features of functional siRNAs^{6,14,15,23,29–35}. A smaller number of algorithms consider the secondary structure and accessibility of the targeted mRNA^{36–38}. The approaches underlying these algorithms range from empirical observations to sophisticated machine learning. After the siRNA sequence selection from the target mRNA sequence, each candidate siRNA is examined for similarity to all other mRNA transcripts that might unintentionally be targeted at a genome-wide level. Most of the siRNA selection algorithms have been combined with a variant of such programs, and the more user-friendly tools are listed in **Table 1** (for a more complete list, see ref. 28). The selected siRNAs can be custom synthesized from four siRNA-licensed reagent suppliers: Ambion, Dharmacon, Qiagen and Sigma Proligo.

Prevalidation of siRNAs

Because the determination of the precise level of gene knockdown for each siRNA is a demanding process, and the assays need to be adapted for newly targeted genes, reporter-based assays have been developed to accelerate the identification of potent siRNAs among various synthesized siRNAs. In these systems, plasmids, which carry the target sequence fused to a reporter

gene and a control gene for normalization, are cointroduced into cells together with the target-specific siRNAs^{14,39,40}. The dual-luciferase-based siCHECK system from Promega is widely used and provides a ranking for siRNA activity within 24 h. The reporter-based activity generally correlates well with the efficacy of depleting the endogenous target (our unpublished observations). The prevalidated siRNAs can then be used to validate the depletion of the endogenous target mRNA, which is discussed in detail in an accompanying review⁴¹.

CONSIDERATIONS FOR SELECTING EFFECTIVE AND SPECIFIC siRNAs

Sequence asymmetry of siRNA duplexes

It has been demonstrated that structurally symmetric (duplexes with symmetric 2-nucleotide (nt) 3' overhangs) but primary sequence-asymmetric (different nucleotides on each end) siRNAs, from which the target-mRNA complementary guide strand has greater propensity to be assembled into the RNA-induced silencing complex (RISC) than the passenger strand, show improved efficacy and specificity^{42,43} (Fig. 1). The same finding emerges from sequence analysis of miRNA precursors and largely explains the asymmetric accumulation of the majority of miRNAs⁴². The asymmetry is determined by the different sequence composition, and the consequent differences in thermodynamic stability and molecular dynamic behavior of the two base-paired ends of an siRNA duplex: the strand with the less stable 5' end, owing to either weaker base-pairing or introduction of mismatches, is favorably or exclusively loaded into RISC⁴⁴. The asymmetry rule has been implemented in many siRNA design algorithms by computing either the A·U base pair content or local free energy at both ends of an siRNA, followed by selection of the duplexes with less stable, (A+U)-enriched 5' end on the guide strand²⁰.

Because the majority of miRNAs start with a 5' uridine, it is also conceivable that 5' uridine-specific interaction contributes to more effective RISC assembly and function beyond the thermodynamic contributions discussed here. Furthermore, miRNA duplexes contain an average of six non-Watson-Crick base pairs distributed over the entire miRNA length, whose contribution to RISC assembly and asymmetry has not been evaluated.

siRNA duplex stability

Most analyzed functional siRNAs had a low-to-medium G+C content ranging between 30% and 52% (refs. 6,31). It has been argued that too low G+C content may destabilize siRNA duplexes and reduce the affinity for target mRNA binding, whereas too high G+C content may impede RISC loading and/or cleavage-product release. Additionally, surveys of functional siRNAs revealed that stable duplexes devoid of internal repeats or palindromes, which may form intrastrand secondary structures, were better silencers^{6,31,45}. An equally likely explanation is that the secondary structure of the target mRNA, which mirrors the predicted guide siRNA secondary structure, interferes with targeting.

Although the overall duplex stability is important, the center of the duplex (positions 9–14 on the guide strand) appears to preferentially have low internal stability^{31,42,46}. It has recently been noticed that miRNAs and siRNAs assemble into RISC by different mechanisms; siRNAs require cleavage of the passenger strand for effective RISC assembly, whereas a mismatched RNase III-processed miRNA duplex does not require passenger strand cleavage⁴⁷. It is conceivable that the central-duplex instability may influence how effectively and to what ratios the RISC complexes with different core components are loaded. Alteration of the structure and stability of siRNA duplexes can also be controlled by incorporation of chemically modified nucleotide analogs. The effects of modifications, however,

Table 1 | Representative siRNA sequence selection web tools

Tools	URLs	Comments	References
siDESIGN	http://www.dharmacon.com/	Scores and ranks candidate siRNAs based on thermodynamic and sequence-related criteria. BLAST search is conducted by default.	6,79
RNAi Designer	https://rnaidesigner.invitrogen.com/rnaexpress/	Ranks candidate siRNAs using a primitive scoring system. BLAST search is automatic and the results are shown.	Proprietary
BIOPREDSi	http://www.biopredsi.org	An artificial neural network-based tool, which was trained with ~2,500 experimentally assessed siRNAs. Analysis of genome-wide specificity is included.	14
Whitehead siRNA Selection server	http://jura.wi.mit.edu/bioc/siRNA	Offers flexibility in defining siRNA sequence patterns and selection of filter functions. Different properties of selected siRNAs are calculated, including thermodynamic values, polymorphisms are identified and the results of configurable BLAST search and filtering are shown. The user can sort the output in various ways and balance decisions.	30
siDE	http://side.bioinfo.ochoa.fib.es/	Developed for high-throughput applications of siRNAs using several published algorithms for efficacy prediction and a nonredundant database for specificity analysis.	80
siSearch	http://sisearch.cgb.ki.se/	The kernel algorithm focuses primarily on energy features of effective siRNAs. Alternative algorithms are also implemented and integrated in the tool. siSearch is expandable to include newly discovered rules.	31
Sirna	http://sfold.wadsworth.org/sirna.pl	Sequence selection tool, which incorporates the target accessibility in the evaluation. No specificity analysis.	36
siRNA design software	http://www.cs.hku.hk/~sirna	Candidate siRNAs proposed by various previously developed sequence selection tools are classified based on target accessibility.	38

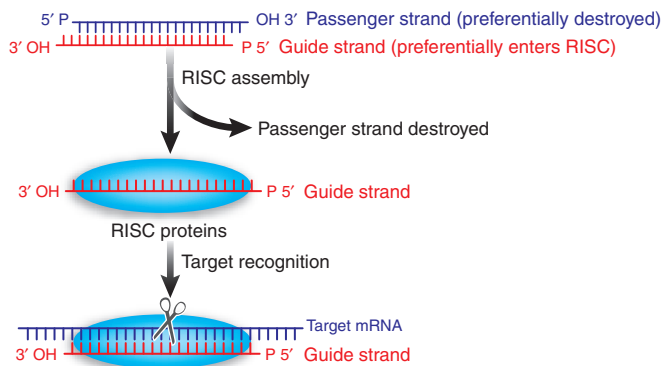


Figure 1 | A scheme for siRNA-mediated gene silencing. The primary sequence asymmetry of duplex determines which strand is preferentially assembled into RISC.

are dependent on the position and the sequence context, and general rules are not yet available^{2,8,48}.

It is interesting to mention that imperfectly paired siRNA duplexes composed of target mRNA-complementary and partially palindromic or partially complementary single-stranded siRNAs have also been used successfully⁴⁹. These siRNA duplexes are solely composed of two fully target-complementary guide strands that are sufficiently complementary to each other to form stable duplexes with characteristic 3' overhanging ends. The silencing efficiencies of guide-only siRNA duplexes are comparable to prototypical fully paired passenger-guide duplex siRNAs, even though guide-only siRNA duplexes may contain a substantial number of non-Watson-Crick and G-U wobble base pairs.

Target accessibility

It has been argued that local secondary structures (short stem-loops) in target mRNAs might restrict the accessibility of RISC, and attenuate or abolish siRNA efficacy^{37,50–53}. A major obstacle in assessing target-accessibility is the lack of tools that reliably predict mRNA secondary structure, setting aside the fact that mRNA is present inside cells as ribonucleoprotein complex of unknown composition. Several algorithms have been developed, and filtering of potentially inaccessible target sequences has been shown to improve functional siRNA selection^{36–38,51}.

Sequence characteristics

Several sequence analyses of siRNAs have independently identified single nucleotide positional preferences, which we will summarize using the guide strand as reference^{6,14,23,29,32–35} (Fig. 2): (i) U or A at position 1; (ii) C or G (C is more common) at position 19; (iii) A+U richness between positions 1 and 7; (iv) A or U (A is more common) at position 10; (v) other motifs that were overrepresented in one analysis but not others, such as a U at position 17. The first three sequence features correlate with the rule of thermodynamic asymmetry, and the preferred nucleotides on indicated positions may contribute to the bias for selection of

antisense strand. The A or U at position 10 is at the cleavage site and may promote catalytic RISC-mediated passenger strand and substrate cleavage. Other sequence determinants may be involved in steps along the RNAi pathway, such as RISC loading⁵⁴.

In addition to the positional nucleotide preference, certain motifs are commonly avoided in chemically synthesized siRNA duplexes that could affect the synthesis yield, purification or the annealing of siRNA strands. Extended runs of altering G-C pairs (more than 7)³² or runs of more than three guanines are sometimes avoided.

Moreover, in light of the reports that certain siRNAs can activate immune response in a cell- and sequence-dependent manner^{55–57}, it is a prudent measure to filter out siRNA sequences containing putative immunostimulatory motifs in either strand to minimize toxicities and nonspecific silencing effects, especially when siRNAs are selected for *in vivo* and therapeutic use. Alternatively, immunostimulatory side effects can be masked using chemically modified nucleotides^{56,58,59}. It is uncertain if all immunostimulatory RNA motifs have yet been defined.

siRNA length and asymmetric 3' overhang

Conventionally designed siRNAs are 21-mers with symmetric 2-nt 3' overhangs, representing the predominant processing intermediate in the RNAi pathway^{60,61}. It was noticed early that 20–25 nt siRNA duplexes carrying 2-nt 3' overhangs could reach similar gene silencing efficiency in mammalian cell culture experiments^{5,62}, and that expressed or synthetic hairpin RNAs of up to 29 base pairs in length also triggered effective gene silencing⁶³. These observations were followed up more recently, testing synthetic 21–29 nt RNA duplexes with blunt ends, symmetric or asymmetric 2-nt overhangs. These siRNA precursor molecules can silence target genes with similar efficiency to conventional siRNA duplexes^{64–68}. The longer dsRNAs appear to have the advantage that they can be transfected at lower concentrations than conventional siRNAs without loss of gene silencing. But they also appear to be more likely to induce nonspecific responses (including interferon induction) or mediate other effects on cell viability^{67,68}.

Short RNA duplexes composed of single-strands of different length (19/21, 21/23 or 25/27 nt formats) have also been shown to silence mRNAs effectively^{66,69,70}. The single 2-nt 3' overhang present in those duplexes at the 3' end of the guide strand and its presumed

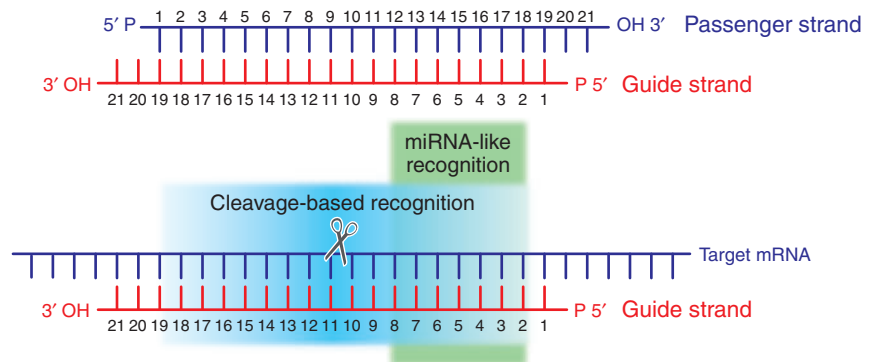


Figure 2 | siRNA and target mRNA structures. (a) Standard siRNA duplex. (b) Target mRNA specificity. The cleavage site is indicated by scissors in the target mRNA. Target recognition and off-target activity can occur in two modes, the catalytic siRNA-guided cleavage reaction requiring extensive complementarity in the region surrounding the cleavage site (blue) and the miRNA-like destabilization of mRNAs requiring pairing of the siRNA 5' end (green).

interaction with the RNAi machinery may contribute to asymmetric RISC assembly. However, the comparison with conventional siRNAs is again complicated by the differences in length of the guide strand and the differences in strength of 5' terminal guide strand base-pairing when the paired region is longer than in conventional siRNA duplexes.

Specificity

Each strand of an siRNA duplex, once assembled into RISC, can guide recognition of fully and partially complementary target mRNAs, referred to as on- and off-targets, respectively. Though sequence asymmetry can be used to bias passenger strand exclusion, chemical methods of preventing passenger-strand use have also been introduced (for example, Dharmacon's ON-TARGET siRNA). For the purpose of this discussion, we will distinguish off-targets into two classes (Fig. 2): (i) those that share contiguous and centrally located sequence complementarity over more than half of the siRNA sequence somewhere within the mRNA sequence⁷¹, and (ii) those that show solely 6 or 7 nucleotides of perfect match preferentially in the 3' UTRs with positions 2–7 or 2–8 (seed region) of the guide siRNA^{9,11,12}. The latter interaction is the major driving force behind endogenous miRNA–target mRNA recognition^{20,72}. Although the off-targets of the latter class are predominant, their actual number identified in microarray analyses was significantly smaller than the number of computationally predicted targets with sequence complementary to the seed region of the guide strand, suggesting that additional specificity determinants remain to be identified^{9,12}.

Furthermore, structural and biochemical studies showed that guide-strand position 1 and the nucleotides at the 3' overhang (positions 20 and 21) have little, if any, contribution to the specificity of target recognition, and that mismatches near the 5' and 3' ends can be tolerated for RISC-guided cleavage if the remaining pairing to the target was unperturbed^{73,74}.

To enforce specificity, the current strategy is to select siRNAs in which the strand(s) entering RISC has some mismatches to all undesired target mRNAs, especially their 3' UTRs. Typically at least three mismatches are recommended between positions 2 and 19 and the mismatches near the 5' end and in the center of the examined strand should be assigned higher significance^{11,71,75,76}. In addition to the position, the identity of the sequence mismatches also influence specificity to a certain extent^{75,77,78}.

Presently most tools use blastn or Smith-Waterman algorithm to remove potential off-targeting siRNAs during the siRNA sequence selection process⁷⁹. In addition to the search method, the quality and completeness of the selected genome-wide mRNA sequence database is also of high importance^{79–81}. The current tools, however, cannot eliminate all the potential off-targets, especially those that contain the short sequence segments complementary to the seed region of the guide strand, and likely discard many potentially functional siRNAs⁹. While improved algorithms are awaited, position-specific chemical modification of the seed-sequence of the guide siRNA can be used to reduce off-target effects⁸². It is therefore important to experimentally control off-target effects or to dilute the off-target effects beyond the detection limit by codelivering several different target-specific siRNAs^{10,41}.

Allele-specific gene silencing

To take advantage of the sequence specificity of RNAi, a prerequisite to achieve allele-specific gene silencing is to identify the most

significant difference between two alleles, which may be as little as a single nucleotide change stemming from mutation or polymorphism⁸³. Placing this sequence discrepancy in the center of an siRNA, at or near the RISC cleavage site seems to be best for discriminating between alleles^{8,76–78,83}. In some cases, introducing an additional mismatch at other positions in the siRNA may improve the allele specificity, as long as the mismatch is tolerated for cleavage⁸³. A limitation of this approach is that the choice of siRNA is restricted, and the siRNA may not be sufficiently effective. In this respect, it is interesting to note that the introduction of a G·U wobble mismatch in the 5' terminal siRNA–mRNA interaction increased the potency of some siRNAs⁷⁵. The efficacy of silencing may also be increased by destabilizing base-pairing at the 5' end of the guide strand following the asymmetry rule⁷⁸.

Alternatively, both alleles can be nondiscriminately silenced by an effective siRNA distant from the polymorphic site, accompanied by ectopic expression of the desired sequence-modified allele refractory to the siRNA⁸⁴. Vectors that simultaneously express transgene and short hairpin RNAs have been developed⁸⁵.

OUTLOOK

In summary, guidelines are available that increase the likelihood of identifying effective and specific siRNAs at the expense of eliminating many potentially functional and specific siRNAs. These guidelines assist in reducing the numbers of siRNAs that need to be experimentally validated to identify potent and specific siRNAs for a given target gene. As reagent manufacturers have recognized the need for constant validation of siRNA knockdown experiments and developed promising lines of reagents, effective siRNAs can be identified at a rapid pace and will soon lead to the ultimate goal of production of validated genome-wide siRNA libraries needed for high-throughput or individual gene silencing experiments.

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