Topic Introduction

RNA Interference and Small RNA Analysis

Chengjian Li and Phillip D. Zamore

Small silencing RNAs have provided powerful reverse genetics tools and have opened new areas of research. This introduction describes the use of RNAi to suppress expression of individual genes for loss-of-function analysis. It also summarizes methods for measuring specific and global changes in small RNA expression, as well as methods to inhibit the function of individual endogenous small RNA species such as miRNAs.

BACKGROUND

RNA interference (RNAi) is the silencing of gene expression in response to double-stranded RNA (dsRNA) that shares sequence with the silenced gene. First observed in plants (Napoli et al. 1990) and subsequently in animals (Guo and Kemphues 1995), RNAi entered practical use when dsRNA was shown to be a highly efficient and reliable silencing trigger (Fire et al. 1998). The RNAi pathway is conserved among nearly all fungi, plants, and animals, allowing its rapid adoption as a reverse genetics tool for functional genomics (Fig. 1; see Box 1 for a glossary of terms).

The first RNAi experiments used exogenously prepared long dsRNA to trigger silencing (Fire et al. 1998; Kennerdell and Carthew 1998; Misquitta and Paterson 1999). Subsequent studies of the mechanism of RNAi revealed a surprisingly complex, ATP-dependent pathway for the stepwise conversion of long dsRNA into functional small RNA–protein complexes. Cleavage of long dsRNA by the RNase III ribonuclease Dicer generates 21- to 23-nt small interfering RNA (siRNA) duplexes bearing 5'-monophosphate and 2-nt, 3'-overhanging ends (Bernstein et al. 2001; Hutvagner et al. 2001; Lee et al. 2004). Next, the siRNA duplex is loaded into an Argonaute protein to form a precursor-RNA-induced silencing complex (pre-RISC). Assembly of pre-RISC requires proteins that sense the relative thermodynamic stability of the ends of the siRNA duplex, thereby establishing which siRNA strand will become the guide (antisense) and which the passenger (sense) strand (Khvorova et al. 2003; Schwarz et al. 2003; Tomari et al. 2004). The passenger strand is removed by the endonucleolytic activity of Argonaute proteins, whereas the guide strand is retained in RISC (Matranga et al. 2005; Rand et al. 2005). Release of the passenger strand converts pre-RISC into mature RISC in which the antisense strand can guide RISC to a complementary site in a target mRNA. Finally, Argonaute in mature RISC cleaves and degrades the target mRNA (Fig. 2).

Long dsRNA is not used to trigger RNAi in mammalian somatic cells, because it induces the interferon response, a set of antiviral responses that includes nonspecific degradation of cellular RNAs and global repression of protein biosynthesis (Minks et al. 1979; Manche et al. 1992). However, the discovery of siRNAs (Hamilton and Baulcombe 1999; Zamore et al. 2000) led to development of synthetic siRNA duplexes that could suppress expression of cognate genes in mammalian somatic cells (Elbashir et al. 2001).

From the Molecular Cloning collection, edited by Michael R. Green and Joseph Sambrook.

© 2019 Cold Spring Harbor Laboratory Press

Cite this introduction as *Cold Spring Harb Protoc*; doi:10.1101/pdb.top097436

FIGURE 1. Summary of RNAi. In the cytoplasm, dsRNAs are cleaved by Dicer to generate siRNA duplexes with 5' monophosphates, 2-nt 3' overhangs. The newborn siRNA duplex is loaded into the Argonaute-containing, RNA-induced silencing complex (RISC). The RISC-loading complex senses the thermodynamic asymmetry of the siRNA duplex and determines the identities of the guide (black) and passenger (blue) strands. The passenger strand is removed by the endonucleolytic activity of Argonaute protein, whereas the guide strand is retained in the mature RISC and directs cleavage of the complementary mRNA targets.

siRNAs are but one of several classes of small RNAs in plants and animals (Ghildiyal and Zamore 2009). The most abundant class of small RNAs is microRNAs (miRNAs). In 1993, Ambros and colleagues discovered the first miRNA, *lin-4*, which controls the developmental timing of *Caenorhabditis elegans* (Lee et al. 1993). Seven years later, the identification of a second miRNA, *let-7*, in bilateral animals including worms, flies, and humans led to the suggestion that miRNAs are evolutionarily conserved regulators of gene expression (Pasquinelli et al. 2000; Reinhart et al. 2000).

miRNAs are ~22-nt endogenous small RNAs processed from ~70-nt hairpin structures in primary miRNA (pri-miRNA) transcripts by either the canonical miRNA pathway or the mirtron pathway (Miyoshi et al. 2010). The difference between these two pathways lies in the mechanism of processing of the pre-miRNA hairpin. In the canonical miRNA pathway, the pre-miRNA hairpin is liberated following cleavage of pri-miRNA by Drosha and its dsRNA-binding partner, Pasha/DGCR8 (Denli et al. 2004; Gregory et al. 2004). In the mirtron pathway, however, the pre-miRNA hairpin is liberated by the RNA splicing machinery and lariat-debranching enzyme (Okamura et al. 2007; Ruby et al. 2007). Subsequently, both types of pre-miRNA hairpins are exported to the cytoplasm by Exportin-5 (Yi et al. 2003; Okamura et al. 2007). In the cytoplasm, Dicer and its partner Loquacious (Loqs)/TRBP/PACT cleave the pre-miRNA hairpin near its loop structure to generate an miRNA/miRNA* duplex (Chendrimada et al. 2005; Förstemann et al. 2005; Jiang et al. 2005; Saito et al. 2005; Lee et al. 2006). The resulting miRNA/miRNA* duplex is loaded into the RISC, where miRNA* is removed. The miRNA guides the RISC to the mRNA targets via complementary base-pairing between the seed region (nucleotides 2–8) of the miRNA and the target site in the 3' UTR and regulates the expression of their endogenous target genes by translational repression or mRNA destabilization (Bartel 2009).

The discovery that miRNAs derive from imperfect hairpins transcribed by RNA polymerase II has been exploited to generate artificial miRNAs—generally referred to as siRNAs. There is as yet no evidence that somatic mammalian cells naturally produce siRNAs. Short hairpin RNAs (shRNAs), mimics of miRNA precursors, can be engineered so that they are converted by Dicer to a functional small RNA duplex within cells. Large collections of such shRNAs are now available for humans and

BOX 1. GLOSSARY

ASOs

Antisense oligonucleotides (ASOs) are short (16–53 nt) synthetic oligonucleotides designed to block the function of RNAs, including microRNAs. They are usually perfectly complementary to the small RNA and contain chemically modified nucleotides or additional flanking sequences to increase their binding affinity and resistance to nucleases in cells.

BLAST

The Basic Local Alignment Search Tool (BLAST) is an algorithm used to calculate the statistical significance of the similarity between nucleotide or amino acid sequences, typically a query sequence and sequence in a database.

cDNA

Complementary DNA is produced by reverse transcriptase using RNA as the template.

CDS

Coding sequence is a region of mature mRNA that is translated into protein, from the start codon to the stop codon.

dsRNA

Double-stranded RNA is an RNA molecule formed by the annealing of two complementary strands. dsRNAs are cleaved by Dicer, giving rise to siRNAs.

Endo-siRNA

Endogenous small interfering RNA is a class of small silencing RNAs found in nearly all eukaryotes.

Exo-siRNA

Exogenous small interfering RNAs are generated either in vitro or in vivo in response to silencing triggers not encoded in the organism's genome. Viral infection or experimental introduction of double-stranded RNA can trigger exo-siRNA production.

miRNA

microRNA is a class of ~22-nt endogenous small RNAs found in plants and animals. miRNAs are processed from ~70-nt hairpin precursors by Dicer. miRNAs regulate their target mRNAs via destabilization of mRNA and translational repression.

piRNA

Piwi-interacting RNA is a class of endogenous small silencing RNAs that are specifically associated with PIWI clade Argonaute proteins in animals. piRNAs do not derive from dsRNA.

RISC

The RNA-induced silencing complex is a small silencing RNA-directed endonuclease, catalyzing the cleavage of the target RNA on the phosphodiester bond at the 5' end of the nucleotide that pairs with the 10th nucleotide of the small RNA guide. Minimally, the RISC comprises a small RNA guide and an Argonaute protein.

RNAi

RNA interference is the sequence-specific silencing of gene expression triggered by double-stranded RNA.

Continued

BOX 1. Continued

shRNA

Short hairpin RNAs are siRNA or miRNA precursors containing 19–29-nt stems and 4–15-nt loops. After transcription, shRNAs are transported from the nucleus to the cytoplasm, where the loop is removed by Dicer cleavage, liberating siRNAs. shRNAs occur naturally, in addition to being used as silencing tools.

shRNAmir

shRNAmir refers to the second generation of shRNA in which an siRNA is embedded in the natural context of an authentic pre-miRNA. Compared with standard shRNAs, shRNAmirs provide more efficient silencing of the target mRNA.

siRNA

Small interfering RNAs are 21- to 23-nt RNA duplexes with 2-nt 3'-overhanging ends. Once loaded into the RISC, one siRNA strand, the passenger strand, is discarded, whereas the other strand, the guide strand, directs the RISC to cleave and degrade a complementary target RNA.

UTR

Untranslated region, the 5' and 3' regions of a mature mRNA that do not code for protein. UTRs contain information for regulation of translation, intracellular localization, and mRNA stability.

mice, enabling whole-genome “RNAi” screens to be conducted using plasmid- or virally expressed shRNA libraries (see Introduction: **Genome-Wide RNA Interference: Functional Genomics in the Postgenomics Era** [Politi and Wajapeyee 2017]). miRNAs are also fascinating endogenous regulators, and technologies to block miRNA function are an important tool to investigate gene function by reverse genetics (see Protocol: **Preparation of Antisense Oligonucleotides to Inhibit miRNA Function**

FIGURE 2. RNA-induced silencing complex (RISC). siRNA duplexes assemble with Argonaute protein to generate pre-RISC containing both siRNAs strands. Subsequently, Argonaute catalyzes cleavage of the passenger (blue) strand. Release of the cleaved passenger strand fragments generates mature RISC, which contains only the guide (black) strand that directs RISC to cleave its cognate RNA target.

[Li and Zamore 2018a], Protocol: **Inhibiting miRNA Function by Antisense Oligonucleotides in Cultured Mammalian Cells** [Li and Zamore 2018b], and Protocol: **Inhibiting miRNA Function by Antisense Oligonucleotides in *Drosophila* S2 Cells** [Li and Zamore 2018c]). Finally, advances in high-throughput sequencing technologies have led to the identification of more and more endogenous small RNAs; high-throughput sequencing is now an indispensable method for studying the biology and biochemistry of small RNAs.

REVERSE GENETICS BY RNAI

Repressing Gene Expression Using Long dsRNA

Introduction of exogenous long dsRNA (see Protocol: **RNAi in *Drosophila* S2 Cells by dsRNA Soaking** [Li and Zamore 2019a] and Protocol: **RNAi in *Drosophila* S2 Cells by siRNA Duplex or dsRNA Transfection** [Li and Zamore 2019b]) or expression of long inverted-repeat RNA transcripts within cells triggers the destruction of the corresponding mRNA in plants, most fungi, nonmammalian animals, and their cultured cells (Fire et al. 1998; Kennerdell and Carthew 1998; Misquitta and Paterson 1999). Long dsRNAs and inverted-repeat RNAs also trigger RNAi in mammalian oocytes and in cultured mammalian cell lines lacking the dsRNA-triggered interferon response, an innate immune defense in which dsRNAs longer than 30 bp activate 2',5'-oligoadenylate synthetase and protein kinase R, which, in turn, activate RNase L and inactivate the α -subunit of eukaryotic initiation factor 2 (eIF2- α), respectively (Minks et al. 1979; Manche et al. 1992). The interferon response induces the nonspecific destruction of mRNAs and global inhibition of protein synthesis, and ultimately triggers apoptosis, making the analysis of specific inhibition of individual genes impossible.

Long dsRNA Design

Typically, 500- to 800-bp-long dsRNAs are used to target exons, preferably coding sequences (CDS) (Hannon 2003). It has been reported that a sequence ≥ 19 nt in length that is shared by the target mRNA and a second mRNA is sufficient to repress expression of the target mRNA (Kulkarni et al. 2006). Thus, it is essential to use algorithms such as dsCheck, E-RNAi, BLAST (see Table 1), or similar computational methods to search for homologies between either the sense or the antisense strand of the dsRNA and cellular mRNAs other than the intended target. Moreover, it is essential to confirm RNAi results with additional, nonoverlapping dsRNAs targeting the same mRNA.

In Vitro Synthesis of Long dsRNAs

In vitro transcription using RNA polymerases from bacteriophages such as T7, SP6, and T3 is an easy and rapid way to prepare long dsRNA for RNAi experiments (see Protocol: **Preparation of dsRNAs for RNAi by In Vitro Transcription** [Li and Zamore 2019c]). In vitro transcription reactions for separate sense or antisense RNA strands are recommended, especially for dsRNAs containing GC-rich regions. Moreover, by this means, the quality of either sense strand or antisense strand RNA can be evaluated separately before annealing them together. Nevertheless, dsRNAs can also be produced from a single in vitro transcription reaction by mixing sense and antisense templates together or using a template with two opposing RNA polymerase promoters.

Plasmid Templates. There are two strategies for making plasmid templates. A DNA fragment corresponding to the selected dsRNA sequence in the sense or antisense orientation can be inserted into the multiple cloning site (MCS) of a plasmid, adjacent to a bacteriophage RNA polymerase promoter. Alternatively, the DNA fragment can be inserted into the MCS of a plasmid in which the insert is flanked by a pair of opposing bacteriophage RNA polymerase promoters. In either method, before in vitro transcription, the plasmid DNA should be linearized with an appropriate restriction enzyme at the end of the sense or antisense insert to ensure production of an RNA transcript of defined length.

TABLE 1. dsRNA and siRNA design tools

Name	URL	Description	Reference
dsRNA design tools			
dsCheck	http://dscheck.rnai.jp	dsRNA selection with off-target analysis	Naito et al. 2005
E-RNAi	http://e-rnai.dkfz.de	Offering both dsRNA and siRNA selection	Arziman et al. 2005
siRNA design tools			
Ambion siRNA Target Finder	http://www.thermofisher.com/us/en/home/brands/invitrogen/ambion.html	Basic siRNA selection guidelines; no specificity check	Ambion
BIOPREDSi		A neural web-based algorithm including genome-wide specificity check	Huesken et al. 2005
BLOCK-iT RNAi Designer	https://rnaidesigner.thermofisher.com/rnaidesigner	A flexible siRNA design tool allowing specificity check	Invitrogen
CAPSID		Antiviral siRNA selection	Lee et al. 2009
DSIR	http://biodev.extra.cea.fr/DSIR/DSIR.html	A linear-model-based algorithm, basic sequences-based siRNA potency prediction	Vert et al. 2006
OptiRNA	http://optima.unl.edu/	Offering mRNA structure prediction but no specificity analysis	Ladunga 2007
siDE		Designed siRNAs for high-throughput application with efficacy prediction and specificity analysis	Santoyo et al. 2005
siDESIGN Center	http://dharmacon.gelifsciences.com/design-center/	Selects siRNAs according to thermodynamic and sequences-based features with toxic motif check and specificity check	Thermo Fisher Scientific (Dharmacon Products)
siDirect2.0	http://siDirect2.RNAi.jp/	Providing siRNA efficacy and novel specificity analysis with off-target analysis	Naito et al. 2009
siDRM		Disjunctive rule merging (DRM)-based approach with toxicity and specificity checks	Gong et al. 2008
Sima	http://sfold.wadsworth.org/cgi-bin/sirna.pl	Target accessibility-based selection without specificity analysis	Ding et al. 2004
siRNA Design Software	http://i.cs.hku.hk/~sirna/software/sirna.php	Multiple-tools-based siRNA selection including target accessibility evaluation	Yiu et al. 2005
siRNA Design Tool	https://www.qiagen.com/no/products/customsirna/customsirnaorder.aspx	Thermodynamic-bias-based selection; no specificity analysis	QIAGEN
siRNA Selection Server	http://sirna.wi.mit.edu/	Provides multiple design parameters including polymorphisms check, specificity check	Yuan et al. 2004
siRNArules 1.0	Downloadable from http://sourceforge.net/projects/sirnarules/	Provides various output formats	Holen 2006
siSearch		Open source JAVA program without flexibility check	
SVM siRNA Design Tool-beta	http://www5.appliedbiosystems.com/tools/siDesign/	Evaluates siRNA efficacy primarily based on the energy features. Selects siRNAs based on the criteria from the analysis of hyperfunctional siRNAs	Chalk et al. 2004 Applied Biosystems

PCR Templates. The small size (19 bp) of the minimal T7, SP6, or T3 bacteriophage promoter allows it to be appended to the 5' end of a forward or reverse PCR primer. PCRs are then performed using a forward primer with the promoter and a reverse primer without the promoter to amplify either sense or antisense templates for in vitro transcription (see Protocol: **Preparation of dsRNAs for RNAi by In Vitro Transcription** [Li and Zamore 2019c]). Alternatively, two promoter-fused primers can be used together to generate a single template with opposing transcription start sites. In vitro transcription with PCR templates can produce a higher yield of RNA than plasmid templates because a higher molar concentration of template can be added without increasing the mass of DNA in the reaction.

Endogenous Expression of Inverted-Repeat dsRNAs

For use in vivo, the long hairpin RNA-expressing cassette is constructed by assembling sense and antisense sequences, typically separated by a spacer, so as to create an inverted repeat (Lee and Carthew 2003). Cloning inverted repeats can be challenging, but using an intron as a spacer can facilitate the process by making the construct more stable in bacteria. Endogenous expression of long hairpin RNAs allows persistent, inducible, or tissue-specific repression of a gene of interest in cell lines or transgenic animals by using RNA polymerase II promoters (e.g., CMV) or binary expression systems (e.g., GAL4/UAS). The main drawback of this approach is that construction of the inverted repeat is time-consuming (see Haley et al. 2008; Ni et al. 2011).

Repressing Specific Gene Activity with siRNAs

The introduction of long dsRNAs triggers specific and robust production of RNAi in mouse oocytes, early embryos, embryonic stem cells, and embryonic carcinoma cells (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000; Billy et al. 2001; Yang et al. 2001), as well as plants, worms, and flies. However, early attempts to elicit RNAi production in mammalian somatic cells using long dsRNAs failed because long dsRNAs as a precursor induce the interferon response. This innate immune response, typically regarded as a response to viral infection, includes the nonspecific degradation of RNA, the global shutdown of protein biosynthesis, and, ultimately, apoptosis. This obstacle was circumvented by using the intermediates in the RNAi pathway, 21- to 23-nt siRNA duplexes with 2-nt 3' overhangs (see Protocol: **RNAi in *Drosophila* S2 Cells by siRNA Duplex or dsRNA Transfection** [Li and Zamore 2019b] and Protocol: **RNAi in Mammalian Cells by siRNA Duplex Transfection** [Li and Zamore 2019d]) (Caplen et al. 2001; Elbashir et al. 2001, 2002), which mimic the natural products of Dicer cleavage of long dsRNAs. Subsequent studies established that endogenous expression of siRNAs and shRNAs provokes specific repression of the target gene at efficiencies comparable to those achieved by the transfection of exogenous siRNAs.

siRNA Design

The selection of siRNA sequences was originally based on empirical experience (Elbashir et al. 2001, 2002). More recently, bioinformatics tools have been developed to design siRNAs (see Table 1), and several databases now catalog experimentally validated siRNAs and shRNAs. It is recommended to search siRNA databases and the scientific literature for previously validated siRNAs before designing new siRNAs. When no validated siRNAs are available, three to five candidate siRNAs should be designed for each target gene (Pei and Tuschl 2006). The main considerations for selecting efficient and specific siRNAs are described below. For detailed, step-by-step guidelines for the design of siRNAs, see Birmingham et al. (2007).

Target Regions. siRNAs are typically designed to target the CDS of mRNAs because anecdotal reports suggest that the CDS is easier to target and should be less polymorphic than noncoding sequences. However, the 3' untranslated region (UTR) can also be used when it is hard to find a suitable siRNA-binding site in the CDS or when trying to distinguish between two genes bearing the same coding sequence but distinct 3' UTRs. The 5' UTR and the splice junctions are typically excluded

because they may be bound by intracellular protein complexes such as the translational initiation machinery or the exon junction complex.

Length and Overhang Structure. Canonical siRNA duplexes are 21 nt long and contain 2-nt, 3'-overhanging "tails" on both ends, which mimic the predominant products made by Dicer in vivo, although 20- to 25-nt-long siRNA duplexes with 2-nt, 3'-overhanging ends show efficacy comparable to that of canonical siRNAs. RNA duplexes larger than 30 bp can induce the interferon response in mammalian cells. Some RNA duplexes longer than 23 bp may induce the interferon response in a cell-type-specific manner (Reynolds et al. 2006).

Thermodynamic Asymmetry. The siRNA strand that is loaded into the RISC is called the "guide strand" (Fig. 2). The siRNA strand whose 5' end is more loosely paired (i.e., less thermodynamically stable) is selected by the RNAi machinery to be the guide (Khvorova et al. 2003; Schwarz et al. 2003). Incorporation of the desired guide strand into the RISC can be enhanced by including a single mismatch or G:U wobble at its 5' end. Conversely, chemical modification (Nykanen et al. 2001; Chen et al. 2008) can be used to block incorporation of the passenger strand—that is, the strand antisense to the guide and sense to the target mRNA—into the RISC.

GC Content and Nucleotide Preference. Most analyses of siRNA design suggest that functional siRNAs should lack palindromic sequences or internal repeats and contain 30%–52% GC. Palindromic sequences or internal repeats may form secondary structures that impair RISC loading as well as target binding (Patzel et al. 2005). Lower or higher GC content may impair separation of the two siRNA strands, slowing RISC loading. Moreover, overly strong binding of mature RISC to a target mRNA may hinder the release of cleavage products, slowing enzyme turnover (Haley and Zamore 2004; Tang and Zamore 2004). A multifactorial analysis of the properties of highly functional siRNAs suggested that guide strand positions 1–7 should be U or A, position 10 should ideally be A or otherwise U, and position 19 should be G or C (Pei and Tuschl 2006). Such "rules" are predicted to favor loading of the intended guide strand, ensure moderate affinity for the target, and promote multiple rounds of target cleavage.

RNAi-Mediated Off-Target Effects. siRNA-mediated off-targeting is the concentration-dependent, unintended repression of gene expression by either the guide or passenger strand of an siRNA duplex (Fig. 3; Jackson et al. 2006a). siRNA-mediated off-targeting occurs when siRNAs act like endogenous miRNAs, which bind their mRNA targets by pairing through their "seed sequence": nucleotides 2–7 or 2–8 of the small RNA guides (Lim et al. 2005; Lin et al. 2005; Birmingham et al. 2006). Several strategies to reduce off-target effects are currently used. Although homology searches (e.g., BLASTn or the Smith–Waterman algorithm) to eliminate potential off-targeting siRNAs are widely used, such a strategy is unlikely to eliminate most off-target effects, as fortuitous 6- and 7-nt seed matches to cellular mRNAs are unavoidable. Decreasing the concentration of each siRNA by pooling multiple, independent siRNAs targeting the same desired mRNA has been proposed to improve specificity (Kittler et al. 2007), but this claim remains to be rigorously established. Sequence-independent chemical modification at position 2 of the guide-strand siRNA similarly seeks to improve efficacy by reducing the binding affinity of the siRNA—both for the intended target and undesirable off-target mRNAs (Jackson et al. 2006b). Perhaps the only proven method to improve specificity is to design siRNAs that load the correct strand into the functional RNAi enzyme complex. Alternatively the passenger strand can be chemically modified to prevent it from acting in the RNAi pathway (Elmén et al. 2005).

Innate Immune Responses and Toxicity. In mammals, the presence of G-rich and U-rich sequence motifs, such as GUCCUCAA or UGUGU, in an siRNA is likely to activate the cellular innate immune pathway via Toll-like receptors (Hornung et al. 2005; Judge et al. 2005; Marques and Williams 2005; Sioud 2005). Moreover, other immunostimulatory determinants remain to be identified, as some G-rich and U-rich siRNAs do not elicit immune responses, whereas some siRNAs that lack either the GUCCUCAA or UGUGU motif are immunostimulatory. A comprehensive comparison of 176 randomly selected siRNAs identified UGGC as a toxic motif leading to cell death (Fedorov et al. 2006). Known immunostimulatory and toxic motifs can be eliminated computationally. Alternatively, chemical modifications, such as locked nucleic acid (LNA) (Fig. 4) and 2'-O-methyl nucleotide modifications on such motifs can be used to suppress innate immune stimulation (Judge et al. 2006).

FIGURE 3. siRNA-mediated off-targeting. When an siRNA fully matches its intended target, it guides RISC to cleave the target RNA target, a process typically referred to as RNAi. However, siRNAs can also act like cellular miRNAs, which bind their RNA targets through partial base-pairing. Such miRNA-like interactions lead to fortuitous off-target repression of unintended mRNAs.

In Vitro Preparation of siRNAs

Chemically Synthesized siRNAs. Synthetic siRNAs are widely used because of their high yield and purity. Synthetic siRNAs also allow a variety of chemical modifications that increase siRNA stability, reduce off-target effects, and/or block innate immune activation. Many companies, including Ambion, Thermo Scientific Dharmacon, QIAGEN, and Sigma-Aldrich Prologo, provide high-quality synthetic siRNAs. Before being introduced into cells, the synthetic sense and antisense siRNAs are annealed together to form siRNA duplexes in vitro (see Protocol: **Preparation of siRNA Duplexes** [Li and Zamore 2019e]).

Enzymatic Production of siRNAs from Long dsRNAs. siRNA duplexes can be prepared by enzymatic digestion of in vitro-transcribed long dsRNAs with recombinant Dicer or bacterial RNase III (Yang et al. 2002). Compared with synthetic siRNAs, enzymatic digestion of a long dsRNA generates a diverse population of siRNAs, increasing the likelihood of generating functional

FIGURE 4. Chemical structure of a locked nucleic acid. LNA is a nucleotide analog with a methylene 2'-O,4'-C linkage, which restricts the ribose sugar pucker to C3'-endo ("North"), the geometry found in an A-form RNA helix. Incorporation into DNA of an LNA nucleotide at every third position changes a B-form helix to an A-form helix, increasing its thermal stability (3°C–8°C/LNA nucleotide). The increased thermal stability (T_m) of LNA-substituted oligonucleotides makes them ideal as short primers and probes (i) to detect small RNA expression and (ii) as antisense oligonucleotides (ASOs) to block small RNA function.

siRNAs. However, the chief liability of siRNAs generated by in vitro “dicing” is that experiments using them are harder to control. Ideally, control siRNAs should not share the same seed sequence as any of the experimental siRNAs. Because the actual siRNAs generated by in vitro dicing are unknown, such precise controls are not possible. Consequently, it may be impossible to establish that a specific phenotype derives from a reduction in expression of the desired target mRNA rather than an unintended off-target gene.

In Vitro Transcription. siRNAs can be generated by in vitro transcription using synthetic DNA oligonucleotide templates containing bacteriophage promoter sequences. Typically, such approaches use nuclease or ribozyme digestion to ensure the production of defined ends and/or a 5' monophosphate or hydroxyl terminus. This approach allows the rapid production of many different siRNAs in an inexpensive fashion but runs the risk that contaminating triphosphorylated RNAs will trigger the innate immune response (Kim et al. 2004).

Expression of siRNA, shRNA, and shRNAmir in Cells

DNA-templated expression of siRNAs, shRNAs, and shRNAmirs (miRNA-adapted shRNAs) within cells can induce transient, persistent, or inducible gene silencing, facilitating systemic analyses of loss-of-function phenotypes.

DNA-Based Expression of siRNAs. In the DNA-based expression of the siRNA approach, the sense and antisense strands of the siRNAs are transcribed from separate RNA polymerase III promoters within the same plasmid construct. After transcription, intermolecular pairing between the sense and antisense strands produces the siRNA duplexes, triggering RNAi (Lee et al. 2002; Miyagishi and Taira 2002). Hybridization of the two siRNA strands within the nucleus is likely to be inefficient, as is their transport to the cytoplasm; shRNAs and similar strategies have largely superseded this approach.

shRNAs. shRNAs are transcribed as single RNA molecules, like precursor miRNAs (pre-miRNAs), which contain 19- to 29-nt stems and 4- to 15-nt loops. After transcription, shRNAs are transported from the nucleus to the cytoplasm, where the loop is removed by Dicer cleavage to liberate siRNAs (Castanotto et al. 2002; McManus et al. 2002; Paddison et al. 2002). Two strategies have been used to express shRNAs: (1) PCR-derived shRNA-expression cassettes and (2) shRNA-expression plasmids or viral vectors. PCR-derived shRNA expression cassettes avoid cloning procedures and allow the rapid production of multiple shRNAs. They are mainly used to screen functional shRNAs. PCR-derived shRNA-expression cassettes only transiently induce knockdown because they lack selectable antibiotic resistance markers. In contrast, shRNA-expression vectors capable of integrating into the genome or of replicating stably as episomes can be used both to express shRNAs transiently and to generate transgenic RNAi cell lines or animals, allowing systematic study of the consequences of gene loss across developmental stages and tissue types.

shRNAmirs. Currently, shRNAmirs are constructed by substituting the stem sequences of human pre-miR-30 with the desired siRNA sequences (Fig. 5). Consequently, a siRNA is embedded into the natural context of an authentic pre-miRNA, which enables efficient cleavage of the siRNA by Drosha and Dicer, and, finally, an enhanced incorporation of the siRNA guide into mature RISC. Compared with standard shRNAs, shRNAmirs provide more efficient reduction of their targets (perhaps 12-fold). Therefore, shRNAmirs are referred to as “second-generation shRNAs” (Zeng and Cullen 2002, 2003; Zeng et al. 2003; Silva et al. 2005).

ANALYSIS OF SMALL RNAs

Measuring Small RNA Expression

Northern Hybridization and RNase Protection

Denaturing polyacrylamide gel-based northern hybridization (see Protocol: **Analysis of Small RNAs by Northern Hybridization** [Li and Zamore 2018d]) is the method used most widely to assay the size

FIGURE 5. An shRNAmir is constructed by substituting the stem sequences of human pre-miR-30 with the desired siRNA sequences. The resulting shRNAmir contains a 19-nt loop and 125-nt flanking sequences of the primary transcript of miR-30, which enhance the efficacy of RNAi. (Adapted by permission from Macmillan Publishers from Silva et al. 2005.)

and the abundance of a small RNA. Most miRNAs are readily detected by using 5' ^{32}P -radiolabeled DNA oligonucleotide probes. StarFire oligonucleotide probes bear multiple [α - ^{32}P] radiolabels at their 3' ends, providing a greater sensitivity by increasing the specific activity of the probe (Behlke et al. 2000). LNA oligonucleotide probes enhance detection by increasing the signal-to-noise ratio of hybridization, producing a more than 10-fold increase in sensitivity; however, LNA oligonucleotides can be costly (Válóczi et al. 2004). In the standard small RNA northern protocol, small RNAs are cross-linked to the nylon membrane using ultraviolet (UV) irradiation. Alternative methods using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-mediated chemical cross-linking have been reported with a 25- to 50-fold increase in sensitivity (Pall et al. 2007; Pall and Hamilton 2008). RNase protection can be more sensitive than northern hybridization, but we advise against this method because it does not provide reliable information regarding the length and abundance of isoforms of a small RNA (Sijen et al. 2001).

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Traditional quantitative RT-PCR cannot measure the expression of small RNAs because their short length does not provide sufficient space for two primers. Traditional quantitative RT-PCR, however, can be used to quantify primary miRNAs (pri-miRNAs), which are typically as long as mRNAs. Since the first report of detection of miRNA precursors (Schmittgen et al. 2004), three novel strategies have been developed to detect miRNAs using quantitative PCR: (1) The 3' end of the miRNA is extended by polyadenylation, and then the elongated miRNA is reverse transcribed with a poly(T) adapter bearing a 3' degenerate anchor and a 5' universal tag. Finally, cDNA is amplified using miRNA-specific forward and universal reverse primers with SYBR Green detection (Shi and Chiang 2005). This strategy allows the relative quantification of multiple miRNAs from a single reverse transcription reaction. (2) The first-strand cDNA is synthesized by an miRNA-specific RT primer with a 5' universal tag and then amplified by an miRNA-specific, LNA-modified primer and a universal primer with SYBR Green detection (Raymond et al. 2005). (3) The first-strand cDNA is synthesized by an miRNA-specific stem-looped RT primer, and then the cDNA is amplified by an miRNA-specific forward primer with a 5' tag and a universal reverse primer in the presence of a specific TaqMan probe (Chen et al. 2005). Compared with miRNA-specific RT primers, the base-stacking of the stem-loop RT primers enhances the thermal stability of the miRNA-RT primer duplexes and thus increases the

specificity and sensitivity of the RT reaction. The 5' tag on the small RNA-specific primer is used to normalize its T_m , allowing further improvements of primer specificity and sensitivity. The TaqMan small RNA assay (Applied Biosystems) is widely used to analyze miRNAs as well as siRNAs. Currently, it can quantify small RNA from as little as 1–10 ng of total RNA with more than a six-log dynamic range.

Microarray Detection of Small RNAs

Small RNA microarrays have a much higher throughput than northern hybridization and are widely used to profile the expression of small RNAs, particularly miRNAs (Krichevsky et al. 2003; Esquela-Kerscher and Slack 2004; Liu et al. 2004; Nelson et al. 2004). Probe normalization and miRNA labeling are the critical considerations for sufficient specificity and sensitivity on small RNA microarrays. To achieve a uniform hybridization condition, the T_m values between probes and miRNAs can be normalized by either incorporating modified nucleotides, such as LNA (Castoldi et al. 2006), or adjusting the length of probes (Baskerville and Bartel 2005). In addition, direct labeling of miRNAs with fluorescent dye has been widely applied to avoid the bias introduced by reverse transcription and PCR amplification. The chief drawback of microarrays is that they can only be used to study annotated small RNAs or to validate those small RNAs predicted from bioinformatic analyses. Moreover, standard microarrays are not available for endogenous siRNAs (endo-siRNAs), exogenous siRNAs (exo-siRNAs), or Piwi-interacting RNAs (piRNAs). The enormous species diversity of these small RNAs (Ghildiyal and Zamore 2009) may exceed the number of probes available using even the largest microarray platform.

In Situ Hybridization to Detect Small RNA Expression Patterns

By using 12- to 22-nt LNA-modified DNA probes, in situ hybridization allows detection of small RNAs in particular cells, tissues, and organs, as well as whole embryos. Recently, an additional fixation procedure was introduced to prevent the loss of miRNAs from tissues by an EDC-mediated cross-link between the 5' phosphate of an miRNA and an amino group from nearby proteins (Pena et al. 2009). Current in situ hybridization methods can detect either miRNA precursors or mature miRNAs but cannot distinguish between the two (Wienholds et al. 2005; Kloosterman et al. 2006; Obernosterer et al. 2007).

Small RNA Cloning and Sequencing

Small RNA cloning and sequencing are powerful tools for identifying new small RNAs. In the past 8 years, a variety of strategies have been formulated to improve the identification of small RNAs bearing different 5'- or 3'-end chemical structures, but the basic principles are similar for all. These include sequential ligation of small RNAs to a 3'- and a 5'-end linker; then RT-PCR amplification to produce a small RNA cDNA library; and finally, DNA sequencing analysis. Initially, small RNA cDNA libraries were cloned into plasmids as concatamers and analyzed by traditional Sanger DNA sequencing (Djikeng et al. 2001; Lagos-Quintana et al. 2001). These days they are read by massively parallel, high-throughput sequencing, which provides an enormous increase in sequencing depth and a reduction in cost (Aravin et al. 2006; Girard et al. 2006; Lau et al. 2006; Brennecke et al. 2007; Ghildiyal et al. 2008; Li et al. 2009). Because the sequencing frequency ("reads") of a small RNA is approximately proportional to its abundance, high-throughput sequencing also allows relative quantification of small RNA expression within the same sample or across different samples, if a thoughtful normalization strategy is applied.

Inhibition of Small RNA Function with Antisense Oligonucleotides

Since 2004, antisense oligonucleotides (ASOs) have been a powerful tool for studying the loss of function of individual miRNAs in cultured cell lines, *Drosophila*, zebrafish, and mice, as well as

nonhuman primates. ASOs also hold promise for therapeutics (Hutvagner et al. 2004; Meister et al. 2004; Esau 2008; Horwich and Zamore 2008; Petri et al. 2009). Typical effective ASOs are 16- to 53-nt synthetic oligonucleotides bearing various chemical modifications on the base, the ribose sugar, the phosphodiester backbone, or the termini. ASOs longer than miRNAs contain a central region complementary to their miRNA targets and an equal number of flanking sequences on both sides. Longer ASOs show enhanced capability of repressing miRNA function. Modified nucleotides include 2'-O-fluoro, 2'-O-methyl, 2'-O-methoxyethyl, 2'-deoxy, and LNA; and backbone modifications include phosphorothioate and peptide nucleic acids (Fabani and Gait 2008). These modifications protect ASOs from cellular nucleases and increase their binding affinity and specificity. Moreover, terminal modifications, such as cholesterol and cell-penetrating peptides, have been shown to facilitate the delivery of ASOs to cells. Preparation of ASOs and their use to inhibit miRNA function in cultured mammalian cells and in *Drosophila* S2 cells are described in Protocol: **Preparation of Antisense Oligonucleotides to Inhibit miRNA Function** (Li and Zamore 2018a), Protocol: **Inhibiting miRNA Function by Antisense Oligonucleotides in Cultured Mammalian Cells** (Li and Zamore 2018b), and Protocol: **Inhibiting miRNA Function by Antisense Oligonucleotides in *Drosophila* S2 Cells** (Li and Zamore 2018c).

ACKNOWLEDGMENTS

We thank Klaus Förstemann, Jennifer Broderick, Michael Horwich, Vasily Vagin, Megha Ghildiyal, and Stefan Ameres for their efforts in developing the protocols described in this introduction and members of the Zamore laboratory for helpful discussions and comments on the manuscript.

REFERENCES

- Aravin A, Gaidatzis D, Pfeffer S, Lagos-Quintana M, Landgraf P, Iovino N, Morris P, Brownstein MJ, Kuramochi-Miyagawa S, Nakano T, et al. 2006. A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* 442: 203–207.
- Arziman Z, Horn T, Boutros M. 2005. E-RNAi: A web application to design optimized RNAi constructs. *Nucleic Acids Res* 33: W582–W588.
- Bartel DP. 2009. MicroRNAs: Target recognition and regulatory functions. *Cell* 136: 215–233.
- Baskerville S, Bartel DP. 2005. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA* 11: 241–247.
- Behlke MA, Dames SA, McDonald WH, Gould KL, Devor EJ, Walder JA. 2000. Use of high specific activity StarFire oligonucleotide probes to visualize low-abundance pre-mRNA splicing intermediates in *S. pombe*. *BioTechniques* 29: 892–897.
- Bernstein E, Caudy AA, Hammond SM, Hannon GJ. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409: 363–366.
- Billy E, Brondani V, Zhang H, Müller U, Filipowicz W. 2001. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc Natl Acad Sci* 98: 14428–14433.
- Birmingham A, Anderson EM, Reynolds A, Ilesley-Tyree D, Leake D, Fedorov Y, Baskerville S, Maksimova E, Robinson K, Karpilow J, et al. 2006. 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Methods* 3: 199–204.
- Birmingham A, Anderson E, Sullivan K, Reynolds A, Boese Q, Leake D, Karpilow J, Khvorova A. 2007. A protocol for designing siRNAs with high functionality and specificity. *Nat Protoc* 2: 2068–2078.
- Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon GJ. 2007. Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* 128: 1089–1103.
- Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA. 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci* 98: 9742–9747.
- Castanotto D, Li H, Rossi JJ. 2002. Functional siRNA expression from transfected PCR products. *RNA* 8: 1454–1460.
- Castoldi M, Schmidt S, Benes V, Noerholm M, Kulozik AE, Hentze MW, Muckenthaler MU. 2006. A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). *RNA* 12: 913–920.
- Chalk AM, Wahlestedt C, Sonnhhammer EL. 2004. Improved and automated prediction of effective siRNA. *Biochem Biophys Res Commun* 319: 264–274.
- Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, et al. 2005. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 33: pe179. doi: 10.1093/nar/gni178.
- Chen PY, Weinmann L, Gaidatzis D, Pei Y, Zavolan M, Tuschl T, Meister G. 2008. Strand-specific 5'-O-methylation of siRNA duplexes controls guide strand selection and targeting specificity. *RNA* 14: 263–274.
- Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R. 2005. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* 436: 740–744.
- Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. 2004. Processing of primary microRNAs by the Microprocessor complex. *Nature* 432: 231–235.
- Ding Y, Chan CY, Lawrence CE. 2004. Sfold web server for statistical folding and rational design of nucleic acids. *Nucleic Acids Res* 32: W135–W141.
- Dijkeng A, Shi H, Tschudi C, Ullu E. 2001. RNA interference in *Trypanosoma brucei*: Cloning of small interfering RNAs provides evidence for retroposon-derived 24–26-nucleotide RNAs. *RNA* 7: 1522–1530.
- 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: 494–498.
- Elbashir SM, Harborth J, Weber K, Tuschl T. 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 26: 199–213.
- Elmén J, Thonberg H, Ljungberg K, Frieden M, Westergaard M, Xu Y, Wahren B, Liang Z, Ørum H, Koch T, et al. 2005. Locked nucleic

- acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res* 33: 439–447.
- Esau CC. 2008. Inhibition of microRNA with antisense oligonucleotides. *Methods* 44: 55–60.
- Esquela-Kerscher A, Slack FJ. 2004. The age of high-throughput microRNA profiling. *Nat Methods* 1: 106–107.
- Fabani MM, Gait MJ. 2008. miR-122 targeting with LNA/2'-O-methyl oligonucleotide mixmers, peptide nucleic acids (PNA), and PNA-peptide conjugates. *RNA* 14: 336–346.
- Fedorov Y, Anderson EM, Birmingham A, Reynolds A, Karpilow J, Robinson K, Leake D, Marshall WS, Khvorova A. 2006. Off-target effects by siRNA can induce toxic phenotype. *RNA* 12: 1188–1196.
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391: 806–811.
- Förstemann K, Tomari Y, Du T, Vagin VV, Denli AM, Bratu DP, Klattenhoff C, Theurkauf WE, Zamore PD. 2005. Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol* 3: pe236. doi: 10.1371/journal.pbio.0030236.
- Ghildiyal M, Zamore PD. 2009. Small silencing RNAs: An expanding universe. *Nat Rev Genet* 10: 94–108.
- Ghildiyal M, Seitz H, Horwich MD, Li C, Du T, Lee S, Xu J, Kittler EL, Zapp ML, Weng Z, et al. 2008. Endogenous siRNAs derived from transposons and mRNAs in *Drosophila* somatic cells. *Science* 320: 1077–1081.
- Girard A, Sachidanandam R, Hannon GJ, Carmell MA. 2006. A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature* 442: 199–202.
- Gong W, Ren Y, Zhou H, Wang Y, Kang S, Li T. 2008. siDRM: An effective and generally applicable online siRNA design tool. *Bioinformatics* 24: 2405–2406.
- Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R. 2004. The Microprocessor complex mediates the genesis of microRNAs. *Nature* 432: 235–240.
- Guo S, Kempthues KJ. 1995. *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* 81: 611–620.
- Haley B, Zamore PD. 2004. Kinetic analysis of the RNAi enzyme complex. *Nat Struct Mol Biol* 11: 599–606.
- Haley B, Hendrix D, Trang V, Levine M. 2008. A simplified miRNA-based gene silencing method for *Drosophila melanogaster*. *Dev Biol* 321: 482–490.
- Hamilton AJ, Baulcombe DC. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286: 950–952.
- Hannon GJ. 2003. *RNAi: A guide to gene silencing*, pp. vii, 436. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Holen T. 2006. Efficient prediction of siRNAs with siRNARules 1.0: An open-source JAVA approach to siRNA algorithms. *RNA* 12: 1620–1625.
- Hornung V, Guenther-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, Noronha A, Manoharan M, Akira S, de Fougerolles A, et al. 2005. Sequence-specific potent induction of IFN- α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med* 11: 263–270.
- Horwich MD, Zamore PD. 2008. Design and delivery of antisense oligonucleotides to block microRNA function in cultured *Drosophila* and human cells. *Nat Protoc* 3: 1537–1549.
- Huesken D, Lange J, Mickanin C, Weiler J, Asselbergs F, Warner J, Meloon B, Engel S, Rosenberg A, Cohen D, et al. 2005. Design of a genome-wide siRNA library using an artificial neural network. *Nat Biotechnol* 23: 995–1001.
- Hutvagner G, McLachlan J, Pasquinelli AE, Bálint E, Tuschl T, Zamore PD. 2001. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 293: 834–838.
- Hutvagner G, Simard MJ, Mello CC, Zamore PD. 2004. Sequence-specific inhibition of small RNA function. *PLoS Biol* 2: pe98. doi: 10.1371/journal.pbio.0020098.
- Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, Guo J, Johnson JM, Lim L, Karpilow J, Nichols K, et al. 2006a. Position-specific chemical modification of siRNAs reduces “off-target” transcript silencing. *RNA* 12: 1197–1205.
- Jackson AL, Burchard J, Schelter J, Chau BN, Cleary M, Lim L, Linsley PS. 2006b. Widespread siRNA “off-target” transcript silencing mediated by seed region sequence complementarity. *RNA* 12: 1179–1187.
- Jiang F, Ye X, Liu X, Fincher L, McKearin D, Liu Q. 2005. Dicer-1 and R3D1-L catalyze microRNA maturation in *Drosophila*. *Genes Dev* 19: 1674–1679.
- Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. 2005. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat Biotechnol* 23: 457–462.
- Judge AD, Bola G, Lee AC, MacLachlan I. 2006. Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. *Mol Ther* 13: 494–505.
- Kennerdell JR, Carthew RW. 1998. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* 95: 1017–1026.
- Khvorova A, Reynolds A, Jayasena SD. 2003. Functional siRNAs and miRNAs exhibit strand bias. *Cell* 115: 209–216.
- Kim DH, Longo M, Han Y, Lundberg P, Cantin E, Rossi JJ. 2004. Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. *Nat Biotechnol* 22: 321–325.
- Kittler R, Surendranath V, Heninger AK, Slabicki M, Theis M, Putz G, Franke K, Caldarelli A, Grabner H, Kozak K, et al. 2007. Genome-wide resources of endoribonuclease-prepared short interfering RNAs for specific loss-of-function studies. *Nat Methods* 4: 337–344.
- Kloosterman WP, Wienholds E, de Bruijn E, Kauppinen S, Plasterk RH. 2006. In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat Methods* 3: 27–29.
- Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS. 2003. A microRNA array reveals extensive regulation of microRNAs during brain development. *RNA* 9: 1274–1281.
- Kulkarni MM, Booker M, Silver SJ, Friedman A, Hong P, Perrimon N, Mathey-Prevot B. 2006. Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays. *Nat Methods* 3: 833–838.
- Ladunga I. 2007. More complete gene silencing by fewer siRNAs: Transparent optimized design and biophysical signature. *Nucleic Acids Res* 35: 433–440.
- Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. 2001. Identification of novel genes coding for small expressed RNAs. *Science* 294: 853–858.
- Lau NC, Seto AG, Kim J, Kuramochi-Miyagawa S, Nakano T, Bartel DP, Kingston RE. 2006. Characterization of the piRNA complex from rat testes. *Science* 313: 363–367.
- Lee YS, Carthew RW. 2003. Making a better RNAi vector for *Drosophila*: Use of intron spacers. *Methods* 30: 322–329.
- Lee RC, Feinbaum RL, Ambros V. 1993. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 75: 843–854.
- Lee NS, Dohjima T, Bauer G, Li H, Li MJ, Ehsani A, Salvaterra P, Rossi J. 2002. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* 20: 500–505.
- Lee YS, Nakahara K, Pham JW, Kim K, He Z, Sontheimer EJ, Carthew RW. 2004. Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* 117: 69–81.
- Lee Y, Hur I, Park SY, Kim YK, Suh MR, Kim VN. 2006. The role of PACT in the RNA silencing pathway. *EMBO J* 25: 522–532.
- Lee HS, Ahn J, Jun EJ, Yang S, Joo CH, Kim YK, Lee H. 2009. A novel program to design siRNAs simultaneously effective to highly variable virus genomes. *Biochem Biophys Res Commun* 384: 431–435.
- Li C, Zamore PD. 2018a. Preparation of antisense oligonucleotides to inhibit miRNA function. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot097527.
- Li C, Zamore PD. 2018b. Inhibiting miRNA function by antisense oligonucleotides in cultured mammalian cells. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot097535.
- Li C, Zamore PD. 2018c. Inhibiting miRNA function by antisense oligonucleotides in *Drosophila* S2 cells. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot097543.
- Li C, Zamore PD. 2018d. Analysis of small RNAs by northern hybridization. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot097493.
- Li C, Zamore PD. 2019a. RNAi in *Drosophila* S2 cells by dsRNA soaking. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot097477.
- Li C, Zamore PD. 2019b. RNAi in *Drosophila* S2 cells by siRNA duplex or dsRNA transfection. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot097485.

- Li C, Zamore PD. 2019c. Preparation of dsRNAs for RNAi by in vitro transcription. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot097469.
- Li C, Zamore PD. 2019d. RNAi in mammalian cells by siRNA duplex transfection. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot097451.
- Li C, Zamore PD. 2019e. Preparation of siRNA duplexes. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot097444.
- Li C, Vagin VV, Lee S, Xu J, Ma S, Xi H, Seitz H, Horwich MD, Syrzycka M, Honda BM, et al. 2009. Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. *Cell* 137: 509–521.
- Lim LP, Lau NC, Garrett-Engle P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433: 769–773.
- Lin X, Ruan X, Anderson MG, McDowell JA, Kroeger PE, Fesik SW, Shen Y. 2005. siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. *Nucleic Acids Res* 33: 4527–4535.
- Liu CG, Calin GA, Meloon B, Gamlie N, Sevignani C, Ferracin M, Dumitru CD, Shimizu M, Zupo S, Dono M, et al. 2004. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc Natl Acad Sci* 101: 9740–9744.
- Manche L, Green SR, Schmedt C, Mathews MB. 1992. Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol Cell Biol* 12: 5238–5248.
- Marques JT, Williams BR. 2005. Activation of the mammalian immune system by siRNAs. *Nat Biotechnol* 23: 1399–1405.
- 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: 607–620.
- McManus MT, Petersen CP, Haines BB, Chen J, Sharp PA. 2002. Gene silencing using micro-RNA designed hairpins. *RNA* 8: 842–850.
- Meister G, Landthaler M, Dorset Y, Tuschl T. 2004. Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA* 10: 544–550.
- Minks M, West DK, Benveniste S, Baglioni C. 1979. Structural requirements of double-stranded RNA for the activation of 2',5'-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. *J Biol Chem* 254: 10180–10183.
- Misquitta L, Paterson BM. 1999. Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): A role for nautilus in embryonic somatic muscle formation. *Proc Natl Acad Sci* 96: 1451–1456.
- Miyagishi M, Taira K. 2002. U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol* 20: 497–500.
- Miyoshi K, Miyoshi T, Siomi H. 2010. Many ways to generate microRNA-like small RNAs: Non-canonical pathways for microRNA production. *Mol Genet Genomics* 284: 95–103.
- Naito Y, Yamada T, Matsumiya T, Ui-Tei K, Saigo K, Morishita S. 2005. dsCheck: Highly sensitive off-target search software for double-stranded RNA-mediated RNA interference. *Nucleic Acids Res* 33: W589–W591.
- Naito Y, Yoshimura J, Morishita S, Ui-Tei K. 2009. siDirect 2.0: Updated software for designing functional siRNA with reduced seed-dependent off-target effect. *BMC Bioinformatics* 10: p392. doi: 10.1186/1471-2105-10-392.
- Napoli C, Lemieux C, Jorgensen R. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* 2: 279–289.
- Nelson PT, Baldwin DA, Searce LM, Oberholtzer JC, Tobias JW, Mourelatos Z. 2004. Microarray-based, high-throughput gene expression profiling of microRNAs. *Nat Methods* 1: 155–161.
- Ni JQ, Zhou R, Czech B, Liu LP, Holderbaum L, Yang-Zhou D, Shim HS, Tao R, Handler D, Karpowicz P, et al. 2011. A genome-scale shRNA resource for transgenic RNAi in *Drosophila*. *Nat Methods* 8: 405–407.
- Nykanen A, Haley B, Zamore PD. 2001. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* 107: 309–321.
- Obernosterer G, Martinez J, Alenius M. 2007. Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. *Nat Protoc* 2: 1508–1514.
- Okamura K, Hagen JW, Duan H, Tyler DM, Lai EC. 2007. The mirtron pathway generates microRNA-class regulatory RNAs in *Drosophila*. *Cell* 130: 89–100.
- Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* 16: 948–958.
- Pall GS, Hamilton AJ. 2008. Improved northern blot method for enhanced detection of small RNA. *Nat Protoc* 3: 1077–1084.
- Pall GS, Codony-Servat C, Byrne J, Ritchie L, Hamilton A. 2007. Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. *Nucleic Acids Res* 35: pe60. doi: 10.1093/nar/gkm112.
- Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Müller P, et al. 2000. Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA. *Nature* 408: 86–89.
- Patzel V, Rutz S, Dietrich I, Köberle C, Scheffold A, Kaufmann SH. 2005. Design of siRNAs producing unstructured guide-RNAs results in improved RNA interference efficiency. *Nat Biotechnol* 23: 1440–1444.
- Pei Y, Tuschl T. 2006. On the art of identifying effective and specific siRNAs. *Nat Methods* 3: 670–676.
- Pena JT, Sohn-Lee C, Rouhanifard SH, Ludwig J, Hafner M, Mihailovic A, Lim C, Holloch D, Berninger P, Zavolan M, et al. 2009. miRNA in situ hybridization in formaldehyde and EDC-fixed tissues. *Nat Methods* 6: 139–141.
- Petri A, Lindow M, Kauppinen S. 2009. MicroRNA silencing in primates: Towards development of novel therapeutics. *Cancer Res* 69: 393–395.
- Politi K, Wajapeyee N. 2017. Genome-wide RNA interference: Functional genomics in the postgenomics era. *Cold Spring Harb Protoc* doi: 10.1101/pdb.top097550.
- Rand TA, Petersen S, Du F, Wang X. 2005. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* 123: 621–629.
- Raymond CK, Roberts BS, Garrett-Engle P, Lim LP, Johnson JM. 2005. Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. *RNA* 11: 1737–1744.
- Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. 2000. The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403: 901–906.
- Reynolds A, Anderson EM, Vermeulen A, Fedorov Y, Robinson K, Leake D, Karpilow J, Marshall WS, Khvorova A. 2006. Induction of the interferon response by siRNA is cell type- and duplex length-dependent. *RNA* 12: 988–993.
- Ruby JG, Jan CH, Bartel DP. 2007. Intronic microRNA precursors that bypass Drosha processing. *Nature* 448: 83–86.
- Saito K, Ishizuka A, Siomi H, Siomi MC. 2005. Processing of pre-microRNAs by the Dicer-1-Loquacious complex in *Drosophila* cells. *PLoS Biol* 3: pe235. doi: 10.1371/journal.pbio.0030235.
- Santoyo J, Vaquerizas JM, Dopazo J. 2005. Highly specific and accurate selection of siRNAs for high-throughput functional assays. *Bioinformatics* 21: 1376–1382.
- Schmittgen TD, Jiang J, Liu Q, Yang L. 2004. A high-throughput method to monitor the expression of microRNA precursors. *Nucleic Acids Res* 32: e43. doi: 10.1093/nar/gnh040.
- Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. 2003. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115: 199–208.
- Shi R, Chiang VL. 2005. Facile means for quantifying microRNA expression by real-time PCR. *BioTechniques* 39: 519–525.
- Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, Plasterk RH, Fire A. 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* 107: 465–476.
- Silva JM, Li MZ, Chang K, Ge W, Golding MC, Rickles RJ, Siolas D, Hu G, Paddison PJ, Schlabach MR, et al. 2005. Second-generation shRNA libraries covering the mouse and human genomes. *Nat Genet* 37: 1281–1288.
- Sioud M. 2005. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *J Mol Biol* 348: 1079–1090.
- Svoboda P, Stein P, Hayashi H, Schultz RM. 2000. Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development* 127: 4147–4156.
- Tang G, Zamore PD. 2004. Biochemical dissection of RNA silencing in plants. *Methods Mol Biol* 257: 223–244.

C. Li and P.D. Zamore

- Tomari Y, Matranga C, Haley B, Martinez N, Zamore PD. 2004. A protein sensor for siRNA asymmetry. *Science* **306**: 1377–1380.
- Válczi A, Hornyik C, Varga N, Burgán J, Kauppinen S, Havelda Z. 2004. Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Res* **32**: pe175. doi: 10.1093/nar/gnh171.
- Vert JP, Foveau N, Lajaunie C, Vandenbrouck Y. 2006. An accurate and interpretable model for siRNA efficacy prediction. *BMC Bioinformatics* **7**: p520. doi: 10.1186/1471-2105-7-520.
- Wianny F, Zernicka-Goetz M. 2000. Specific interference with gene function by double-stranded RNA in early mouse development. *Nat Cell Biol* **2**: 70–75.
- Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, Horvitz HR, Kauppinen S, Plasterk RH. 2005. MicroRNA expression in zebrafish embryonic development. *Science* **309**: 310–311.
- Yang S, Tutton S, Pierce E, Yoon K. 2001. Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. *Mol Cell Biol* **21**: 7807–7816.
- Yang D, Buchholz F, Huang Z, Goga A, Chen CY, Brodsky FM, Bishop JM. 2002. Short RNA duplexes produced by hydrolysis with *Escherichia coli* RNase III mediate effective RNA interference in mammalian cells. *Proc Natl Acad Sci* **99**: 9942–9947.
- Yi R, Qin Y, Macara IG, Cullen BR. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17**: 3011–3016.
- Yiu SM, Wong PW, Lam TW, Mui YC, Kung HF, Lin M, Cheung YT. 2005. Filtering of ineffective siRNAs and improved siRNA design tool. *Bioinformatics* **21**: 144–151.
- Yuan B, Latek R, Hossbach M, Tuschl T, Lewitter F. 2004. siRNA Selection Server: An automated siRNA oligonucleotide prediction server. *Nucleic Acids Res* **32**: W130–W134.
- Zamore PD, Tuschl T, Sharp PA, Bartel DP. 2000. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**: 25–33.
- Zeng Y, Cullen BR. 2002. RNA interference in human cells is restricted to the cytoplasm. *RNA* **8**: 855–860.
- Zeng Y, Cullen BR. 2003. Sequence requirements for micro RNA processing and function in human cells. *RNA* **9**: 112–123.
- Zeng Y, Yi R, Cullen BR. 2003. MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc Natl Acad Sci* **100**: 9779–9784.

Cold Spring Harbor Protocols

RNA Interference and Small RNA Analysis

Chengjian Li and Phillip D. Zamore

Cold Spring Harb Protoc; doi: 10.1101/pdb.top097436

Email Alerting Service

Receive free email alerts when new articles cite this article - [click here](#).

Subject Categories

Browse articles on similar topics from *Cold Spring Harbor Protocols*.

[Molecular Biology, general](#) (1293 articles)
[RNA](#) (317 articles)
[RNA Interference \(RNAi\)/siRNA](#) (123 articles)
[RNA, general](#) (269 articles)

To subscribe to *Cold Spring Harbor Protocols* go to:
<http://cshprotocols.cshlp.org/subscriptions>