Published in final edited form as:
Methods Mol Biol. 2010 ; 629: 141–158. doi:10.1007/978-1-60761-657-3_10.

Short Hairpin RNA (shRNA): Design, Delivery, and Assessment of Gene Knockdown

Chris B. Moore, **Elizabeth H. Guthrie**, **Max Tze-Han Huang**, and **Debra J. Taxman**

Abstract

Shortly after the cellular mechanism of RNA interference (RNAi) was first described, scientists began using this powerful technique to study gene function. This included designing better methods for the successful delivery of small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) into mammalian cells. While the simplest method for RNAi is the cytosolic delivery of siRNA oligonucleotides, this technique is limited to cells capable of transfection and is primarily utilized during transient in vitro studies. The introduction of shRNA into mammalian cells through infection with viral vectors allows for stable integration of shRNA and long-term knockdown of the targeted gene; however, several challenges exist with the implementation of this technology. Here we describe some well-tested protocols which should increase the chances of successful design, delivery, and assessment of gene knockdown by shRNA. We provide suggestions for designing shRNA targets and controls, a protocol for sequencing through the secondary structure of the shRNA hairpin structure, and protocols for packaging and delivery of shRNA lentiviral particles. Using real-time PCR and functional assays we demonstrate the successful knockdown of ASC, an inflammatory adaptor molecule. These studies demonstrate the practicality of including two shRNAs with different efficacies of knockdown to provide an additional level of control and to verify dose dependency of functional effects. Along with the methods described here, as new techniques and algorithms are designed in the future, shRNA is likely to include further promising application and continue to be a critical component of gene discovery.

Keywords

RNA interference (RNAi); small interfering RNA (siRNA); short hairpin RNA (shRNA); lentivirus; design; delivery; ASC; *Porphyromonas gingivalis*; IL-1β; ELISA; THP1

1. Introduction

In recent years, the use of RNA interference (RNAi) has emerged as a powerful tool for the study of gene function in mammalian cells. The mechanism of RNAi is based on the sequence-specific degradation of host mRNA through the cytoplasmic delivery of double-stranded RNA (dsRNA) identical to the target sequence (1). Degradation of target gene expression is achieved through an enzymatic pathway involving the endogenous RNA-induced silencing complex (RISC). One strand of the siRNA duplex (the guide strand) is loaded into the RISC with the assistance of Argonaute (Ago) proteins and double-stranded RNA-binding proteins. The RISC then localizes the guide strand to the complementary mRNA molecule, which is subsequently cleaved by Ago near the middle of the hybrid (2). The cleaved mRNA is further degraded by other endogenous nucleases. Likewise, the RISC also plays an important cellular role in inhibiting endogenously derived mRNA through a related micro-RNA (miRNA) mechanism (3).

Several methods of RNAi have evolved over time, with the simplest approach involving the transfection of chemically synthesized short interfering RNA oligonucleotides (siRNAs) directly into the cytosol (*see* **Chapters 4**, **5,** and **9**). While the delivery of siRNAs can be achieved in many cell types, variable transfection efficiencies have limited siRNA-mediated RNAi to only those cells capable of transfection. Another form of RNAi involves the use of short hairpin RNAs (shRNAs) synthesized within the cell by DNA vector-mediated production. Like siRNAs, shRNAs may be transfected as plasmid vectors encoding shRNAs transcribed by RNA pol III or modified pol II promoters, but can also be delivered into mammalian cells through infection of the cell with virally produced vectors. While siRNA delivers the siRNA duplex directly to the cytosol, shRNAs are capable of DNA integration and consist of two complementary 19–22 bp RNA sequences linked by a short loop of 4–11 nt similar to the hairpin found in naturally occurring miRNA. Following transcription, the shRNA sequence is exported to the cytosol where it is recognized by an endogenous enzyme, Dicer, which processes the shRNA into the siRNA duplexes (*see* **Chapter 7**). Like the exogenously delivered synthetic siRNA oligonucleotides, this endogenously derived siRNA binds to the target mRNA and is incorporated into the RISC complex for target-specific mRNA degradation (4).

Although siRNA and shRNA ultimately utilize a similar cellular mechanism (RISC), the choice of which method to use depends on several factors such as cell type, time demands, and the need for transient versus stable integration. There are a variety of reagents available for siRNA design and synthesis. Therefore, the efficiency of knockdown for each siRNA sequence can be rapidly determined and, in fact, there are several commercial sources for siRNA which have been functionally validated. In addition, siRNA delivery has benefited from the plethora of transfection reagents already in existence, yielding a potentially high level of gene silencing with minimal cellular toxicity. An increasing concern with siRNA, however, is the apparent increased probability of incurring off-target effects due to the high concentration of cytoplasmic siRNA. Another significant disadvantage to siRNA oligonucleotide delivery is that as the cells divide, the siRNA concentration becomes diluted, thereby rendering the generation of a long-term cell line with the desired target gene knockdown unfeasible. shRNA, on the other hand, may be used to generate stable knockdown cell lines, thereby eliminating the need for multiple rounds of transfection and greatly increasing reproducibility of results. However, the creation of a stable shRNA cell line is a time-consuming task as the construct preparation and the selection of shRNA-positive cells by drug resistance or fluorescent markers may take months. With this said, many cells cannot be transfected with siRNA at high levels, especially primary and non-adherent cells, such as immune cells and non-dividing cells. Transfection efficiency is a major issue for siRNA since incomplete transfection produces incomplete knockdown which may fail to ablate the function of the protein. For most untransfectable cells, adenoviral, retroviral, or lentiviral-based shRNA technology remains the only viable technology for the successful delivery of RNAi. For these reasons, this article focuses primarily on methodologies applicable to shRNA, though many of the suggestions may also be useful for siRNA.

The proper selection of a target sequence for a given gene of interest remains one of the most critical components of successful gene knockdown regardless of the RNAi methodology. Although target RNAi sequences have been constructed from 19 to 27 bp, most data on effective sequence selection involve the design of 19 bp targets. While there is no guarantee of effective gene silencing for a given siRNA until experimentally proven, numerous algorithms have been designed to predict these 19 bp targets with a nucleotide composition thought to confer the highest efficacy (5–8). In addition, as new algorithms are designed frequently, it is imperative that one should use the most modern design method available for selecting a target site. Protocol 3.1 provides general guidelines for how to use

the available algorithms to design shRNA knockdowns and appropriate controls. A minimum of two target sequences should be designed for each gene, in order to increase the likelihood that at least one sequence results in significant gene knockdown. Additionally, two successful knockdowns can also provide a useful control for off-target knockdowns since it is statistically unlikely that different sequences will produce the same off-target knockdowns. Figures 10.1 and 10.2 give an example of how two shRNAs with differing efficacies can also be extremely useful in showing dose dependency for functional studies.

Once the target sites are selected, shRNA vectors must be constructed. Two basic methods for constructing shRNA vectors, oligonucleotide-based cloning and PCR-based cloning, have been provided elsewhere (9). Specific cloning vectors and protocols for constructing shRNA vectors for appropriate applications are also available commercially from multiple biotechnology companies. For any newly constructed shRNA vector, it is essential to confirm the sequence of the hairpin since single-base mismatches within the target can alter specificity. Though many shRNA plasmids will sequence sufficiently under standard sequencing conditions, a number of shRNAs will be problematic due to the intrinsic secondary structure of the hairpin. In **Section 3.2**, some basic steps are provided for sequencing even the most problematic shRNA hairpins. These recommendations are based on a detailed analysis of the effects of sequencing additives alone or in combination (6).

There are multiple methods of introducing siRNA and shRNA into cells. The method of choice depends on whether transient or stable expression is desired and the model system. Lentiviral-mediated transduction provides a convenient method of introducing shRNA into dividing or non-dividing cells and, in general, is less toxic to the cells than adenoviral-mediated trans-duction. **Sections 3.3** and **3.4** describe effective methods for preparation of lentiviral particles and transduction into adherent or non-adherent cells.

Once the shRNA plasmid is prepared and introduced into the cells, it is necessary to confirm effective knockdown. As an example of knockdown determination, Protocol 3.5 and Fig. 10.1 describe the confirmation of knockdown of the innate immunity adaptor molecule, ASC (10, 11), by quantitative PCR analysis. These knockdowns are based on two shRNAs against ASC that have been previously produced by our laboratory using a murine stem cell-based retroviral vector, pHSPG (6, 12, 13). For the current study, the ASC shRNAs were re-cloned into a lentiviral vector, FG12 (14), and knockdown in this system was tested. Details of the PCR assay used to verify ASC knockdown by the FG12-shRNA vector are provided (**Section 3.5**).

If the gene targeted for knockdown has a known biological or physiological function, a functional assay can also be extremely useful in testing the efficacy of an shRNA. Protocol 3.6 illustrates how a functional assay can verify knockdown. In this example, ASC is known to play an integral role in the function of the inflammasome, a multiprotein cytosolic complex required for the cleavage and activation of IL-1β (10, 11). To test the function of the ASC shRNAs, we have performed ELISAs for IL- 1β following infection of THP1 human monocytic cells with the periodontitis-associated pathogen, *Porphyromonas gingivalis* (Fig. 10.2). Similar to previous results using the pHSPG vector (6, 12, 13), the shRNAs produced in FG12 are functional in blocking IL-1β expression. The effects of the knockdowns are dose-dependent, providing further verification of the findings and a convenient additional control for the experiment (Fig. 10.2).

2. Materials

2.1. Selecting RNA Target Sites and Sequencing Through shRNA Hairpins

1. To select shRNA target sites and corresponding controls, internet access is required.
2. Dimethyl sulfoxide.
3. BigDye Terminator v1.1 cycle sequencing ready reaction mix (Applied Biosystems).
4. ABI Prism dGTP BigDye terminator ready reaction mix. (Applied Biosystems)
5. Betaine.
6. 1X PCRx Enhancer (Invitrogen; included with Pfx DNA Polymerase).
7. Centri-Sep 96-well spin plates (Princeton Separations). 8. 3730 series DNA Analyzer (Applied Biosystems).

2.2. Packaging shRNA-Encoding Lentivirus

1. Lentiviral vector, envelop vector (e.g., pMDL or other Gag-Pol vector), and packaging vector(s) (encoding VSV-G and Rev genes).
2. 2X BES-buffered saline (BBS)—0.5 M BES, 150 mM Na_2HPO_4, pH 6.95.
3. 1 M $CaCl_2$ dissolve in sterile water and stored at −20°C.
4. 293T cells.
5. Dulbecco's modified Eagle's medium. 6. Fetal calf serum (FCS).

2.3. Stable Transduction of Adherent or Non-adherent Cells with shRNA-Encoding Lentivirus

1. Viral supernatant from **Section 3.3**.
2. Cells to be transduced.
3. Growth media for the cells. Usually RPMI, 10% FCS or DMEM, 10% FCS, depending on the cell type.
4. 2.0 mL round-bottom microcentrifuge tubes.
5. 8.0 mg/mL polybrene stock solution dissolved in sterile water and stored at −20°C.
6. Table top centrifuge (for adherent cells) or swing-bucket microcentrifuge (for non-adherent cells).
7. Appropriate agent for drug selection for lentiviral vectors that contain a drug-resistance gene.

2.4. Confirmation of Knockdown of ASC by Real-Time PCR Analysis

1. RNeasy purification kit (Qiagen).
2. 100 μM oligo$(dT)_{15}$.
3. 10 mM dNTP mix: 10 mM each dATP, dCTP, dGTP, and Analysis dTTP.
4. MMLV reverse transcriptase.
5. 5X FS Buffer (Invitrogen; included with MMLV).

6. 0.1 M DTT (included with MMLV).
7. RNaseOUT (Invitrogen) or RNaseIN (Promega).
8. PCR grade water.
9. PCR pipette tips.
10. PCR primers (*see* **Section 3.5**).
11. 384-Well PCR plates.
12. Optical adhesive film.
13. 2X SYBR Green PCR Master Mix.
14. AB Prism 7700 thermocycler.

2.5. ELISA to Confirm Functional Knockdown of ASC

1. Stably transduced control and knockdown cells.
2. *P. gingivalis*, or other immunostimulatory agent.
3. RPMI 1640 supplemented with 10% FCS.
4. Human IL-1β ELISA set.

3. Methods

3.1. Selecting shRNA Target Sites and Corresponding Controls

1. Determine whether the gene of interest has one or multiple splice variants. Decide whether you want to target all potential forms of a gene or specific splice variants. Select exons for targeting accordingly (*see* Note 1).
2. Select several potential target sites within the exon or exons of interest within your gene or within the 5′ or 3′ UTR depending on which splice forms of the gene are to be targeted (*see* Note 2). If the function of an siRNA or shRNA against your gene of interest has been validated commercially or in a publication it may be useful to test the same target site in your system. An siRNA or shRNA that shows efficacy in one cell system in knocking down expression of its target is usually effective in other cell systems.
3. If a validated siRNA target is not available for your exon(s) of choice, you will need to design the shRNA target anew. There are several commercial and non-commercial web sites available for siRNA design (*see* Note 3). Use the most current design algorithm and web resource available. The following steps may or may not be included as an option depending on the algorithm and web resource,

---

[1]One of the most critical considerations in selecting a target site is the consideration of all splice forms of the targeted mRNA. For general knockdown of a gene, the site selected must target every splice form in order to yield interpretable results.

[2]If the appropriate spice variants of the gene are targeted, the position of the target site (UTR, 5′, middle, or 3′) does not appear to have a general effect on the efficacy of a given siRNA or shRNA. However, local mRNA secondary structure has been postulated to play a role for certain genes.

Since it is difficult to predict the local secondary structure effects at this time, it is best to design different shRNAs that target different regions of the gene if possible.

[3]Web sites that offer algorithms for siRNA and shRNA target site selection include http://shRNAdesigner.med.unc.edu, http://www.dharmacom.com, and http://www.ambion.com. In most cases, a target site that is effective for siRNA will also be effective for shRNA. However, there are additional steps involved in the processing of shRNA by Dicer, and the design criteria for shRNA may not be 100% identical to those for siRNA in all cases (6). For this reason we recommend our web algorithm which is specifically based on shRNA design (http://shRNAdesigner.med.unc.edu). Additionally, we have found that effective shRNA target sites may have overlap in which algorithms predict them, and looking for target sites predicted to be efficacious by two or more different algorithms could potentially provide an additional useful strategy.

and their inclusion as design criteria depends upon your specific experimental model:

   a. For shRNAs that are to be expressed from a plasmid or viral vector under control of a pol III promoter, avoid target sequences with runs of four or more A's or T's. These sequences may create potential problems with premature termination during transcription. Some of the common hairpin loop sequences begin with two T nucleotides, and if this is the case, be careful that the target sequence does not end with more than one T (*see* Note 4).

   b. For the design of shRNA that will be expressed from a U6 or 7S K promoter, select target sequences beginning with a G.

4. Perform a BLAST search to eliminate potential target sequences that have a perfect match of 16 nt or more to an off-target gene of the same species (http://www.ncbi.nlm.nih.gov/BLAST/).

5. Eliminate potential target sites that overlap regions of single-nucleotide polymorphisms (SNPs) (http://www.ncbi.nlm.nih.gov/projects/SNP/).

6. Select two or more shRNAs targeting different regions within the same gene that have the fewest amounts of BLAST matches, that do not overlap a region of SNP, and that target different regions within the gene (*see* Note 5).

7. Also design control shRNAs, including a non-targeting shRNA with a fully or partially scrambled targeting sequence (*see* Note 6).

3.2. Sequencing Through shRNA Hairpins

1. Design two sequencing primers, one for either strand of the vector into which the shRNA is cloned. Primers should lie approximately 50–100 bp upstream (for the sense strand) or downstream (for the antisense strand) from the hairpin and be approximately 20 nt in length (*see* Note 7).

2. Set up a standard sequencing reaction (12.5 μL total volume) containing 1X BigDye Terminator v1.1 cycle sequencing ready reaction mix (Applied Biosystems), 0.26 μg of DNA, and 3.75 pmol of sequencing primer. Also add 5% DMSO. This will be sufficient for sequencing through the majority of shRNA hairpins.

3. For more problematic hairpins that do not sequence sufficiently with the conditions in step 2, sequencing reactions may be modified by substituting the standard BigDye Terminator v1.1 chemistry with a mixture of 10 parts BigDye v1.1

---

[4]For one of the most commonly used hairpin loops, based on a naturally occurring miRNA sequence (15), the complete hairpin sequence would read N19-TTCAAGAGA-rN19, where N19 is the target sequence and rN19 is the reverse complement of the target. If the N19 target were to end in two T's, the combination of the two T's from the target sequence together with the first two T's of the loop would constitute four T's in a row, which can comprise a termination signal for RNA pol III.

[5]The use of two different siRNAs of shRNAs provides an extremely useful control against the possible effects of off-target knockdowns since it is statistically unlikely that two siRNAs will have the same off-target knockdowns. Also, since no algorithm can guarantee effective silencing, preparing two or more siRNAs or shRNAs increases the probability of obtaining knockdown. shRNA or siRNA with different extent of silencing also can be useful in verifying dose-dependent functional effects as shown in Figs. 10.1 and 10.2.

[6]Experimental controls can include a mock-infected or mock-transfected sample, an empty vector, an shRNA encoding a scrambled target, or an shRNA targeting another gene entirely. If possible, use at least 2–3 controls. Many researchers prefer a control that is known to encode a functional shRNA against another gene. Genes within entirely different pathways or even of different species may be used. Some examples of common control genes for siRNA or shRNA are firefly luciferase and green fluores-cence protein.

[7]It is helpful to design a sequencing primer for each strand since the secondary structure of the hairpin often makes it selectively more difficult to read through one strand.

chemistry to 1 part ABI Prism dGTP BigDye Terminator ready reaction mix. Also add 0.83 M Betaine and 1X PCRx Enhancer (Invitrogen) to the sequencing reaction for read through of virtually any shRNA hairpin.

4. Perform PCR using the following standard thermal cycler program:

95°C × 3 min
98 °C × 40s
50 °C × 5s
60°C × 4 min
98 °C × 10s
50 °C × 5s for 24 cycles
60°C × 4 min
cool to 4°C

5. Purify the sequencing reactions using Centri-Sep columns or 96-well plates. Run the purified products on a 3730 series DNA Analyzer.

3.3. Packaging shRNA-Encoding Lentivirus

1. Day 0: Plate 293T cells in 10 cm plates in DMEM and 10% FCS and grow overnight. A 175 cm^2 flask at 50% confluency is sufficient for approximately 4–5 plates; however, the plating density will vary depending on the starting confluency and the growth of the cell culture (*see* Note 8).
2. Day 1: Cells should be about 70% confluent and evenly distributed on the plate. Aspirate off the medium and add 5 mL of DMEM without serum.
3. Aliquot the lentiviral vector and packaging vectors into a 5 mL snap cap tube as follows:
    a. 15 μg lentiviral vector.
    b. 5 μg envelop vector (e.g., pMDL or other Gag-Pol vector).
    c. 10 μg packaging vector(s) (*see* Note 9).
    d. Add water to yield 375 μL total volume.
4. Add 125 μL of 1 M $CaCl_2$ to the DNA mixture and vortex.
5. Add 500 μL of 2X BBS to the mixture dropwise, while vortexing, and then incubate the DNA/$CaCl_2$/BBS mixture for 30 min at room temperature.
6. Add the transfection mixture to the plate dropwise. Incubate in a CO_2 incubator (either 5% CO_2 or 3% CO_2) for 2–3 h and then add 0.5 mL serum to the culture. Incubate the cells overnight (*see* Note 10 for steps 6–10 of this protocol and Protocol 3.4).
7. Day 2: Remove the media and replace with 7 mL DMEM and 10% FCS. Return cells to a 5% CO_2 incubator for 40–48 h. Virus production peaks at about 40 h (*see* Note 11).

---

[8] 293T cells should not be used after about passage 15 or if growth slows significantly.
[9] In some packaging systems the VSV-G and Rev genes are combined into one plasmid and in other cases they are separated into two vectors. If the genes are provided in two separate vectors, use 5 μg of each vector.
[10] Viral supernatant is infectious and should be treated with appropriate precautions.
[11] For viruses encoding a fluorescent marker gene, such as GFP, 293T cells can be checked microscopically at this point to ensure transfection efficiency. The cells should be close to 100% GFP positive.

8. Day 4: Collect viral supernatant and filter through a 0.45 μm syringe filter to remove any cells or cell debris. The 293T plates can now be bleached and discarded according to approved biosafety procedures.
9. Use viral supernatant fresh or aliquot and freeze at −80°C for at least 6 months to 1 year.

3.4. Stable Transduction of Adherent or Non-adherent Cells with shRNA-Encoding Lentivirus

1. Plate the cells to be transduced with the shRNA-encoding lentivirus as follows:
   a. For adherent cells, plate cells at approximately 60–70% confluency in a 24-well plate, one well per sample, and allow cells to attach overnight. Remove media and replace with 500 μL fresh growth media per well.
   b. For non-adherent cells, spin down 1×10^6 cells per sample and resuspend in 500 μL growth media in a 2 mL round-bottom microfuge tube.
2. Add 0.5 μL of polybrene to each sample to yield a final concentration of 8 μg/mL.
3. Add 1 mL of viral supernatant (*recall* Note 10).
4. Spin tubes or plates at 2,000 rpm for 1–3 h using a swing-bucket rotor if possible (*see* Note 12).
5. Remove the supernatant and replenish the cells with 1 mL of growth media for adherent cells, or for non-adherent cells replenish with 3 mL of growth media and plate in a 6-well plate in a CO_2 incubator overnight.
6. Repeat steps 1–4 for increased efficiency of infection.
7. Plate cells and culture as needed.
8. Depending on the marker gene in the lentiviral vector, you may be able to use the marker to assess percent transduction. If the virus has the green fluorescent protein (GFP) gene, for example, FACS analysis can be done to correlate GFP positivity and transduction levels. If cells are not 95–100% GFP positive, cell sorting could be done to increase levels of stable shRNA-expressing cells. If a drug resistance marker is contained within the viral vector, drug selection should be set up to eliminate any cells that did not receive the shRNA. For any of these assays it is necessary to wait at least 48–72 h to give the cells a chance to express the stably encoded marker gene.

3.5. Confirmation of Knockdown of ASC by Real-Time PCR (see Notes 13 and [14])

1. Design primers for analysis of mRNA levels by quantitative PCR. Primers should target the same splice forms that the shRNAs target, should span an intron/exon junction if possible (*see* Note 15), should lie approximately 100–150 nt apart, and

[12]Spinoculation may not be essential for all non-adherent cells, but can greatly increase transduction efficiency depending on the cell type. A swing-bucket rotor is more effective than a fixed rotor at concentrating the virus onto the cells.
[13]*See* Fig. 10.1 for an example of a real-time PCR experiment used to verify knockdown of ASC in THP1 cells.
[14]As a confirmation of real-time PCR results, immunoblotting may be used to assess knockdown at the level of the protein. Immunoblots should use either polyclonal antibodies targeted against the entire protein or poly-clonal/monoclonal antibodies that target an epitope within the same splice forms targeted by the siRNA or shRNA.
[15]Designing PCR primers to span an exon/intron junction reduces the possible background SYBR signal from contaminating genomic DNA within the sample. As an alternative, the optional on-the-column DNAse step can be performed during the RNeasy purification procedure.

should have a T_m of approximately 57°C. A primer design program such as Primer Express or Primer Designer 4 can be used to assist in primer design (*see* Note 16).

2. Isolate RNA from knockdown cells on three different days (*see* Note 17). Also isolate RNA from several control cells, for example, untransfected cells, cells stably transfected with an empty vector, cells stably expressing a scrambled target sequence, and shRNA targeting an irrelevant gene. Use an RNeasy purification kit to purify total RNA from approximately 2×10^6 cells.

3. Prepare cDNA as follows:

   a. Combine 1 μL 100 μM oligo(dT), 1 μL 10 mM dNTP mix, and 1 μg of RNA. Add water to yield a final volume of 12 μL.

   b. Heat the mixture for 5 min at 65°C and then incubate on ice for at least 1 min.

   c. Prepare a master mix including the following components per sample (*see* Note 18):

      4 μL FS buffer,

      2 μL 0.1 M DTT,

      1 μL RNaseOUT or RNaseIN,

      1 μL MMLV reverse transcriptase.

   d. Add 8 μL of the master mix to each sample. Mix by pipet-ting up and down and then incubate at 42°C for 90 min.

4. Prepare a 1:10 dilution of the cDNA by combining 3 μL cDNA with 27 μL of PCR grade water. Also prepare a 1:5,000 dilution by combining 2 μL of the 1:10 diluted cDNA with 1 mL of PCR grade water.

5. Pipette 4.2 μL of either the 1:10 dilution of the cDNA for the gene of interest or the 1:5,000 dilution of the cDNA for the 18s rRNA (or other housekeeping gene; *see* Note 19) into duplicate wells of a 384-well plate.

6. Prepare a mastermix of SYBR green mix and primers (*see* Note 18). This should include 5 μL per sample of SYBR mix and 0.8 μL/sample of primer mix (5 μM each of forward and reverse primers from Step 1). Add 5.8 μL of mastermix per well.

7. Run on an AB Prism 7700 instrument (Applied Biosystems) or a similar thermocycler with the following program:

48°C × 5 min
95°C × 10 min

[16]Before using a new set of primers in a quantitative experiment, test the primers as follows: (1) add a dissociation step to the PCR profile and look at the dissociation curve after real-time PCR is performed to be sure that a distinct peak of SYBR activity is apparent; (2) run a titration of four 10-fold dilutions of a positive control cDNA sample and ensure that the SYBR activity accurately reflects the dilutions; and (3) recover the 384-well plate following real-time PCR and run the product of the PCR on an agarose gel. Make sure that the product appears as a discrete band that is approximately 100–150 bp in length and that the relative intensities of the bands in different lanes reflect the relative amounts of cDNA added to the PCR reaction.

[17]We have found that knockdown levels for mRNA can fluctuate from day to day as assayed by real-time PCR. For that reason we assess knockdown on 3 days and calculate an average value.

[18]Prepare enough master mix for all samples plus approximately 10% so that there will be enough to account for any pipetting errors.

[19]We routinely use 18s rRNA as a standard for normalization of cDNA levels. We use the following forward and reverse primers for 18s rRNA (6, 13): FW-CGGCTACCACATCCAAGG; RV-GCTGCTGGCACC-AGACTT. Other housekeeping genes, such as GAPDH or cyclophilin-b, can also be used in place of the 18s gene.

95 °C × 30s

58°C × 30s for 40 cycles

95 °C × 15s

58 °C × 15s dissociation stage

95°C × 15s

8. Determine the relative amounts of mRNA for your gene of interest using the comparative CT method (Applied Biosys-tems). Standardize values to the expression of the endogenous 18s rRNA or another endogenous housekeeping gene (*see* Note 19).

3.6. ELISA Analysis to Confirm Functional Knockdown of ASC (see Note 20)

1. Plate THP1 control and ASC knockdown cells lines at 10^6 cells/mL in a 24-well plate (1 mL/well) in a 37°C CO_2 incubator. **(see** Note 20)
2. Add bacterial or other immunostimulatory agent that is known to activate IL-1β through the inflammasome complex (*see* Note 21).
3. Incubate cells for 2 h (for stronger inducers) to overnight (for weaker inducers) (*see* Note 22). For cells induced by infection with a bacterial pathogen, add antibiotics 1 h following infection to prevent subsequent bacterial growth in the culture.
4. Transfer supernatant to a microcentrifuge tube and spin for 5 min at high speed to remove any cells or cellular debris.
5. Recover 900 μL of supernatant to a new microcentrifuge tube and use immediately in an ELISA experiment or store at −20°C for up to several months.
6. Run the ELISA experiment according to manufacturer's recommendations and calculate IL-1β levels in control and knockdown cells by comparison to standard curve. Use log–log regression analysis as recommended by the manufacturer.

3.7. Future Challenges, Promise, and Scientific Developments

Since its inception, the use of RNAi technology has revolutionized how we perform research on gene function. However, the use of this technology is likely to include further challenges in addition to some exciting new applications. One major challenge is in the design of the RNAi. Although significant progress has been made over the past several years in predicting which siRNA target sequences are most effective in reducing gene expression (5–8, 16), currently the only way to ensure the efficacy of an siRNA is by direct experimentation. As our understanding of RNAi mechanisms improves, it is likely that we will be able to better predict the functionality of each siRNA through the development of more accurate algorithms. These new algorithms should include specific sequence requirements for each target and the ability to predict the putative effects of secondary mRNA structure. Also, the role of off-target knockdowns, including the degradation of non-identical mRNAs through the RISC pathway, as well as miRNA-type inhibition of translational elongation, is continually being understood in more detail. The more complete understanding of off-target knockdowns will further the effective design and implementation of RNAi. Other advances in the design of shRNA will likely include the identification of more effective RNA hairpin

[20] *See* Fig. 10.2 for an example of the application of ELISA to verify ASC knockdown.

[21] 1.0 μg/mL lipopolysaccharide is a common immunostimulant that can be used for inducing inflammasome activation (10, 11); however, there are many inflammasome inducers. In Fig. 10.2 we have chosen to use 10 MOI *P. gingivalis*, an oral pathogen that has previously been shown to induce IL-1β in an ASC-dependent manner (12, 13).

[22] Strong immunostimulants can also promote toxicity, requiring a shortened length of induction.

structures, including modified loop sequences; structural or chemical RNA modifications that can alter the mechanisms of action of the siRNA in a favorable and predictable manner; and the identification of additional proteins that can mimic or modulate the function of the Dicer and RISC complexes. Traditionally, shRNAs have been expressed using pol III promoters since they produce a shorter more predictable transcript; however, recent studies have identified modified pol II transcripts that can increase shRNA expression levels (17, 18). The ability to fine-tune expression levels of shRNAs will be important to the efficient use of shRNA since levels that are too low may not be effective, while levels too high can cause toxicity. The ability to control siRNA expression levels may be especially important for genes involved in cell survival, in which case the identification inducible shRNA promoters should be useful (19, 20).

An additional obvious challenge to RNAi is the effective delivery of siRNA or shRNA. The availability of improved commercial transfection reagents has improved siRNA, but still some cells remain difficult to transfect. Lentivirus and adenovirus have made it possible for cells that are refractory to transfection such as primary cells to become permissive to shRNA. However, as these methods are based on a viral backbone, each method harbors inherent dangers which would limit their use to in vitro studies.

In vivo delivery of RNAi also offers great promise for the future. Since current in vivo gene function studies involve the time-consuming development of transgenic mouse gene knockouts and double knockouts, a successful in vivo RNAi protocol would represent a tremendous step forward in terms of time allocation and likely lead to an explosion of knowledge obtained from such studies. Most current approaches to in vivo RNAi involve the systemic delivery of “naked” siRNAs. These so-called naked siRNAs are only moderately effective in the in vivo knockdown of a gene of interest and mostly are limited to genes expressed within the liver and kidney (21). In addition, since naked siRNAs exhibit poor pharmacokinetics, they are delivered at high concentrations, adding to their expense and putative off-target effects. However, there exists at least one in vivo siRNA delivery transfection reagent (Invivofectamine, Invitrogen, Inc.), which has shown promise in the delivery of much lower concentrations of siRNA to a mouse; however, this reagent remains cost prohibitive to most laboratories and these data remain to be reproduced readily outside of its commercial source (http://invitrogen.cnpg.com/Video/flatFiles/761/index.aspx). In addition, viral vectors encoding shRNAs have shown promise for in vivo delivery; however, most of these studies have utilized adenoviral delivery of shRNA, which has well-known toxic effects in the animal (22). Recently, adeno-associated viral vectors (AAV) have been designed with less toxicity and adequate shRNA delivery (23). Finally, there are numerous ongoing studies focused on virally mediated delivery of shRNA to hematopoietic stem cells (HSC) isolated from a mouse and re-implanted into an irradiated recipient mouse (24). These HSCs have been shown to give rise to cells with stable shRNA; however, the recipient mouse still retains gene expression within stromal cells. Nonetheless, this may prove an effective strategy for in vivo studies of gene expression in cells of an immune origin. Notwithstanding these advances in in vivo RNAi, there are still numerous challenges to methodology and application; however, with every new publication comes the exciting possibility of another breakthrough in RNAi technology which will likely advance this field far beyond what is conceivable today.

Acknowledgments

We thank Dr. David Baltimore for supplying us with FG12. We thank Dr. Jenny Ting for her support and guidance. These studies were supported by grants 1-RO1-DE016326 and 1-U54-AI057157.

References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998; 391:806–811. [PubMed: 9486653]
2. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 2001; 15:188–200. [PubMed: 11157775]
3. Maniataki E, Mourelatos Z. A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev. 2005; 19:2979–2990. [PubMed: 16357216]
4. Kutter C, Svoboda P. miRNA, siRNA, piRNA: knowns of the unknown. RNA Biol. 2008; 5:181–188. [PubMed: 19182524]
5. Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, Ueda R, Saigo K. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucl Acids Res. 2004; 32:936–948. [PubMed: 14769950]
6. Taxman DJ, Livingstone LR, Zhang J, Conti BJ, Iocca HA, Williams KL, Lich JD, Ting JP, Reed W. Criteria for effective design, construction, and gene knockdown by shRNA vectors. BMC Biotechnol. 2006; 6:7. [PubMed: 16433925]
7. Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nat Biotechnol. 2004; 22:326–330. [PubMed: 14758366]
8. Amarzguioui M, Prydz H. An algorithm for selection of functional siRNA sequences. Biochem Biophys Res Commun. 2004; 316:1050–1058. [PubMed: 15044091]
9. Taxman, DJ. siRNA and shRNA design. In: Helliwell, TDC., editor. RNA Interference Methods for Plants and Animals. Vol. 10. CABI; Oxfordshire, UK: 2009. p. 228-253.
10. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, Roose-Girma M, Erickson S, Dixit VM. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature. 2004; 430:213–218. [PubMed: 15190255]
11. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell. 2002; 10:417–426. [PubMed: 12191486]
12. Huang MT, Taxman DJ, Holley-Guthrie EA, Moore CB, Willingham SB, Madden V, Parsons RK, Feather-stone GL, Arnold RR, O'Connor BP, et al. Critical role of apoptotic speck protein containing a caspase recruitment domain (ASC) and NLRP3 in causing necrosis and ASC speck formation induced by Porphyromonas gingivalis in human cells. J Immunol. 2009; 182:2395–2404. [PubMed: 19201894]
13. Taxman DJ, Zhang J, Champagne C, Bergstralh DT, Iocca HA, Lich JD, Ting JP. Cutting edge: ASC mediates the induction of multiple cytokines by Porphyromonas gingivalis via caspase-1-dependent and -independent pathways. J Immunol. 2006; 177:4252–4256. [PubMed: 16982856]
14. An DS, Donahue RE, Kamata M, Poon B, Metzger M, Mao SH, Bonifacino A, Krouse AE, Darlix JL, Baltimore D, et al. Stable reduction of CCR5 by RNAi through hematopoietic stem cell transplant in non-human primates. Proc Natl Acad Sci USA. 2007; 104:13110–13115. [PubMed: 17670939]
15. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science. 2002; 296:550–553. [PubMed: 11910072]
16. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. Cell. 2003; 115:199–208. [PubMed: 14567917]
17. Xia H, Mao Q, Paulson HL, David-son BL. siRNA-mediated gene silencing in vitro and in vivo. Nat Biotechnol. 2002; 20 :1006–1010. [PubMed: 12244328]
18. Denti MA, Rosa A, Sthandier O, De Angelis FG, Bozzoni I. A new vector, based on the PolII promoter of the U1 snRNA gene, for the expression of siRNAs in mammalian cells. Mol Ther. 2004; 10:191–199. [PubMed: 15272480]
19. Gupta S, Schoer RA, Egan JE, Han-non GJ, Mittal V. Inducible, reversible, and stable RNA interference in mammalian cells. Proc Natl Acad Sci USA. 2004; 101:1927–1932. [PubMed: 14762164]

20. Unwalla HJ, Li MJ, Kim JD, Li HT, Ehsani A, Alluin J, Rossi JJ. Negative feedback inhibition of HIV-1 by TAT-inducible expression of siRNA. Nat Biotechnol. 2004; 22:1573–1578. [PubMed: 15568018]

21. Lewis J, Melrose H, Bumcrot D, Hope A, Zehr C, Lincoln S, Braithwaite A, He Z, Ogholikhan S, Hinkle K, et al. In vivo silencing of alpha-synuclein using naked siRNA. Mol Neurodegener. 2008; 3:19. [PubMed: 18976489]

22. Vlachaki MT, Hernandez-Garcia A, Ittmann M, Chhikara M, Aguilar LK, Zhu X, Teh BS, Butler EB, Woo S, Thompson TC, et al. Impact of preimmunization on adenoviral vector expression and toxicity in a subcutaneous mouse cancer model. Mol Ther. 2002; 6:342–348. [PubMed: 12231170]

23. Monahan PE, Jooss K, Sands MS. Safety of adeno-associated virus gene therapy vectors: a current evaluation. Expert Opin Drug Saf. 2002; 1:79–91. [PubMed: 12904163]

24. Bot I, Guo J, Van Eck M, Van Santbrink PJ, Groot PH, Hildebrand RB, Seppen J, Van Berkel TJ, Biessen EA. Lentiviral shRNA silencing of murine bone marrow cell CCR2 leads to persistent knockdown of CCR2 function in vivo. Blood. 2005; 106:1147–1153. [PubMed: 15886324]

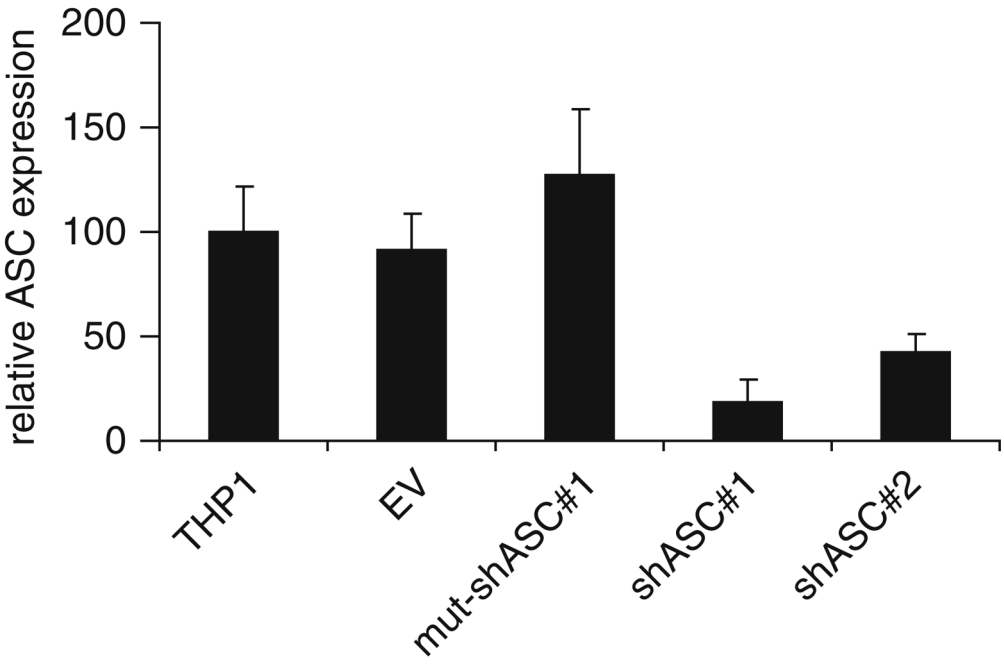

Figure 10.1.

Knockdown of ASC in THP1 cells transduced using lentiviral shRNA vector, FG12 (14). THP1 cells were transduced with lentivirus expressing shRNA against ASC. Our previous studies have shown efficacy for these same shRNAs in reducing ASC expression when expressed using a stem cell virus-based retroviral vector pHSPG (6, 13). Similar to our previous results, the shASCs transduced using FG12 reduce endogenous ASC levels in THP1 monocytic cells by approximately 80% (shASC#1) and 60% (shASC#2). Three control cell lines were also tested for comparison, untransfected THP1 cells (THP1), cells transduced with an empty lentiviral vector (EV), and cells transduced with a lentivirus expressing a scrambled target for shASC#1 (mut-shASC#1). Results represent the averages plus standard deviations of triplicates, are standardized to 18s rRNA expression, and are normalized to an average of 100 in THP1 cells.

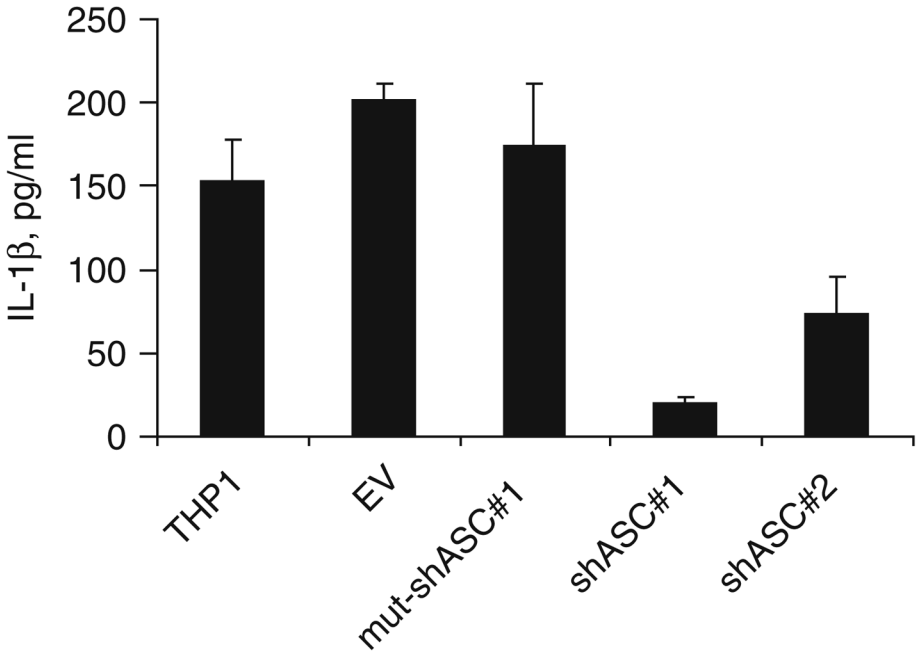

Figure 10.2.

ELISA of IL-1β in control and shASC knockdown cell lines following infection with 10 MOI *Porphyromonas gingivalis*. This figure demonstrates how a functional assay can be used to verify knockdowns. In this case of our protein of interest, ASC, has a well-established role in processing IL-1β following infection with bacteria (10, 11, 13). The reduced IL-1β that is observed for the shRNA cell lines following infection with *P. gingivalis* verifies the knockdowns. Additionally, the experiment shows dose dependency since the shASC#2 is less effective than shASC#1 in knocking down ASC (Fig. 10.1) and also has proportionally less efficacy in reducing IL-1β secretion levels. This figure, therefore, illustrates both the general utility of a functional assay and the advantage of having two different knockdowns of different efficacy to verify dose-dependent functional effects. The use of two shRNAs also provides an additional level of control for studies of ASC function since two shRNAs are statistically unlikely to promote the same off-target knockdowns.