Chapter 9

Transcriptional Gene Silencing Using Small RNAs

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Abstract

RNA interference is a potent gene silencing pathway initiated by short molecules of double-stranded RNA. Small interfering RNAs (siRNAs) with full sequence complementarity to mRNAs induce cleavage of their target transcripts in the cytoplasm. Recent evidence has shown, however, that siRNAs can also function in the nucleus of mammalian cells to affect changes in chromatin structure. When targeted to promoter regions, siRNAs load into the effector protein Argonaute-1 (AGO1) and direct the formation of silent chromatin domains. This mechanism is known as transcriptional gene silencing (TGS), and the development of TGS as a novel therapeutic modality would be applicable to chronic diseases where long-term, heritable silencing of target genes is warranted. Here we discuss how small RNAs can be used to direct TGS in mammalian cells.

Key words: RNAi, siRNA, microRNA, TGS, chromatin, epigenetic, methylation, transcription.

1. Introduction

1.1. RNAi and Transcriptional Gene Silencing

RNA interference (RNAi) is triggered by small RNAs that silence gene expression at the post-transcriptional level *(1)*. Over the past few years, it has become increasingly apparent that components of the RNAi machinery also function in the nucleus of mammalian cells. Small interfering RNAs (siRNAs) and microRNAs (miRNAs) 22 nucleotides in length initiate sequence-specific gene silencing by loading into effector Argonaute (AGO) proteins *(1)*, and when targeted to promoter regions, small RNAs induce transcriptional gene silencing (TGS) through Argonaute-1 (AGO1) and histone methylation *(2)*. Marks of facultative heterochromatin, histone H3 lysine 9 dimethylation (H3K9me2) and histone H3 lysine 27 trimethylation (H3K27me3), are enriched at

John F. Reidhaar-Olson and Cristina M. Rondinone (eds.), *Therapeutic Applications of RNAi: Methods and Protocols*, *vol. 555*

DOI 10.1007/978-1-60327-295-7_9 Springerprotocols.com

promoter regions targeted by small RNAs *(2)* and can be measured using chromatin immunoprecipitation (ChIP) assays. In this chapter, we describe how to induce TGS in mammalian cells through the effective design of promoter-targeting siRNAs and how to assess the efficacy of silencing by examining chromatin marks at the silenced promoter, as well as using nuclear run-on assays.

1.2. Potential Therapeutic Applications of TGS

The use of TGS as a therapeutic modality would be applicable for targeting chronic diseases such as human immunodeficiency virus (HIV) infection and consequent acquired immune deficiency syndrome (AIDS) *(1)*. Targeting the long-terminal repeat (LTR) of HIV-1 with siRNAs has been shown to induce TGS *(3)*. Similar to TGS at endogenous promoters, the HIV-1 LTR exhibits H3K27me3 when targeted by siRNAs in the U3 region of the LTR. Moreover, the chemokine (C-C motif) receptor 5 (CCR5), which serves as a co-receptor for HIV-1, is amenable to TGS using siRNAs *(2)*. Knockdown of CCR5 is therapeutically relevant, since the loss of CCR5 would inhibit R5-tropic HIV-1 from gaining entry into its target cells. The development of therapeutic applications of TGS are still preliminary, however, and require further testing in primary cells before additional progress can be made.

2. Materials

2.1. Design of Promoter-Targeting siRNAs

1. http://www.invitrogen.com/rnai/
2. http://genome.ucsc.edu/

2.2. Assessing Histone Methylation Using ChIP

1. Synthetic siRNAs (Invitrogen).
2. Lipofectamine 2000 (Invitrogen).
3. 1% formaldehyde.
4. 0.125 M glycine.
5. 1X PBS.
6. PMSF.
7. ChIP lysis buffer, 50 mM HEPES at pH 7.5, 140 mM NaCl, 10% Triton X-100, 0.1% Sodium Deoxycholate (NaD), 1/1000 PMSF.
8. Bioruptor sonicator with refrigerated water bath and a rotating 1.5 mL microtube unit (Diagenode).
9. 50% Protein G Agarose slurry (Upstate).
10. Anti-AGO1 (Upstate: 07-599), Anti-H3K9me2 (Upstate: 07-441), and Anti-H3K27me3 (Upstate: 07-449).

11. ChIP lysis buffer high salt, 50 mM HEPES at pH 7.5, 500 mM NaCl, 1% Triton X-100, 0.1% NaD, 1/1000 PMSF.
12. ChIP wash buffer, 10 mM Tris-HCl at pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% NaD, 1 mM EDTA.
13. Elution buffer, 50 mM Tris-HCl at pH 8.0, 1% SDS, 10 mM EDTA.
14. RNase A (10 mg/mL).
15. 5 M NaCl.
16. 0.5 M EDTA.
17. 1 M Tris-HCl at pH 6.5.
18. 10 mg/mL Proteinase K.
19. Phenol/chloroform.
20. iQ SYBR Green Supermix (Bio-Rad).

2.3. Measuring Transcriptional Silencing Using Nuclear Run-On

1. Synthetic siRNAs (Invitrogen).
2. Lipofectamine 2000 (Invitrogen).
3. 1X PBS.
4. 0.5% NP-40 lysis buffer, 10 mM Tris-HCl at pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$.
5. Reaction buffer, 10 mM Tris-HCl at pH 8.0, 5 mM $MgCl_2$, 0.3 mM KCl.
6. 2.5 mM NTP + Biotin-16-UTP mix (Roche).
7. RNA STAT-60 Reagent (Tel-Test).
8. Streptavidin beads (Active Motif).
9. 2X SSC, 15% formamide.
10. 2X SSC.
11. 10 mM EDTA at pH 8.2.

3. Methods

3.1. Design of Promoter-Targeting siRNAs

Use an online siRNA design program, such as Invitrogen's RNAi designer, to enter the promoter sequence of a given gene of interest. Promoter sequences for human genes can be obtained from the UCSC Genome Brower. Select promoter sequences –1 to –200 bp upstream of the known gene transcription start site and enter them into the online siRNA design program. Choose up to four siRNAs that span the promoter region to test their efficacy in triggering TGS of the target gene. The efficacy of siRNA-mediated TGS may be enhanced with increasing proximity to the transcription start site, and several TGS studies *(2–6)* have shown targeting of promoter sequences –1 to –200 bp upstream of transcription start sites (**Table 9.1**). Important considerations for siRNA design include proper antisense or guide strand incorporation in AGO, since recent findings have shown that the antisense

Table 9. 1
Designing promoter-targeting siRNAs

TGS target genes	siRNA target sites
EF1A	–106 to –86
CDH1	–181 to –161
PR	–26 to –7
CCR5	–183 to –163
uPA	–131 to –111

strand alone is sufficient to induce TGS and heterochromatin formation, whereas the sense strand has no effect *(3)*. Furthermore, low levels of sense transcription across the targeted promoter region may be required for TGS to occur at a given gene promoter *(7)*.

3.2. Assessing Histone Methylation Using ChIP

Using siRNAs to target a promoter region induces epigenetic modifications that silence gene expression at the chromatin level. Histone methylation marks H3K9me2 and H3K27me3 are enriched in promoter regions targeted by siRNAs. Two studies have demonstrated that the CDH1 *(4)* and CCR5 *(2)* promoters exhibit increased levels of H3K9me2 when targeted with siRNAs. Additionally, the siRNA-targeted EF1A promoter exhibits both H3K9me2 and H3K27me3 during TGS *(3)*, suggesting that siRNAs might regulate the "histone code" through the recruitment of histone methyltransferases such as the Polycomb group component EZH2 *(2)*. Here we outline how to assess chromatin marks at siRNA-targeted promoters using ChIP, as previously described *(3)*.

1. Transfect cells with control or promoter-specific siRNAs at 50 nM final concentration using Lipofectamine 2000 (*see* **Note 1**).
2. 24–48 h following transfection, crosslink 2×10^7 cells with 1% formaldehyde for 10 min at room temperature (*see* **Note 2**).
3. Stop crosslinking by adding glycine at a final concentration of 0.125 M for 10 min at room temperature.
4. Wash cells twice with cold 1X PBS + 1/1000 PMSF.
5. Harvest cells in 1 mL of ChIP lysis buffer.
6. Incubate for 10 min on ice.
7. Centrifuge samples at $2{,}000 \times g$ for 5 min at 4°C and remove supernatant.
8. Resuspend pellet in 1 mL of ChIP lysis buffer and incubate on ice for 10 min.

9. Sonicate lysates using a Bioruptor sonicator with refrigerated water bath and a rotating 1.5-mL microtube unit for five cycles of 30 s ON and 30 s OFF at HIGH setting (*see* **Note 3**).
10. Centrifuge sonicated samples at 14,000 × *g* for 10 min at 4°C.
11. Remove supernatant and preclear with 60 μL of 50% Protein G Agarose slurry for 1 h at 4°C with rotation.
12. Divide samples and perform immunoprecipitations using 1–5 μg of Anti-AGO1, Anti-H3K9me2, and/or Anti-H3K27me3 overnight at 4°C with rotation, along with no antibody controls.
13. Add 60 μL of 50% Protein G Agarose slurry to each sample for 1 h at 4°C with rotation.
14. Centrifuge at 1,200 × *g* for 3 min at 4°C.
15. Save no antibody control supernatants and use as input control.
16. Perform two washes with 1 mL of ChIP lysis buffer, two washes with 1 mL of ChIP lysis buffer high salt, followed by two washes with 1 mL of ChIP wash buffer.
17. For each wash, incubate samples for 3 min at room temperature on a rotating platform, followed by centrifugation at 1,200 × *g* for 3 min at room temperature.
18. Add 100 μL of elution buffer for 10 min at 65°C, followed by centrifugation at 14,000 × *g* for 3 min at room temperature (repeat Step 18).
19. Reverse crosslinks in eluted samples by adding 1 μL of RNase A and 20 μL of 5 M NaCl and incubate for 4–6 h at 65°C.
20. Add 10 μL of 0.5 M EDTA, 20 μL of 1 M Tris-HCl at pH 6.5, and 2 μL of Proteinase K and incubate for 1 h at 45°C.
21. Recover DNA by phenol/chloroform extraction.
22. Use promoter-specific primers and iQ SYBR Green Supermix to assess the enrichment of chromatin marks using quantitative PCR (qPCR) (*see* **Note 4**).

3.3. Measuring Transcriptional Silencing Using Nuclear Run-On

Here we outline how to measure the extent of transcriptional silencing using nuclear run-on assays, as previously described *(8)*.

1. Transfect cells with control or promoter-specific siRNAs at 50 nM final concentration using Lipofectamine 2000.
2. 24–48 h following transfection, wash 2 × 10^7 cells twice with cold 1X PBS.
3. Harvest cells and lyse on ice in 0.5% NP-40 lysis buffer.
4. Centrifuge at 500 × *g* for 10 min.
5. Remove supernatants.
6. Incubate nuclei in reaction buffer and 2.5 mM NTP + Biotin-16-UTP mix for 45 min at 30°C.

7. Stop transcription reaction by adding RNA-STAT-60 and recover RNA.
8. Isolate biotinylated nascent RNA transcripts by incubation with streptavidin beads for 2 h at room temperature on a rocking platform.
9. Centrifuge at 3,000 × *g* for 3 min to collect beads.
10. Wash once with 2X SSC, 15% formamide for 10 min on a rocking platform.
11. Wash twice with 2X SSC for 5 min on a rocking platform.
12. Elute biotinylated RNA from streptavidin beads in H_2O or 10 mM EDTA at pH 8.2 by incubating at 90°C for 10 min.
13. Use mRNA-specific primers to analyze nascent RNA transcript levels using qRT-PCR.

4. Notes

1. Analogous to traditional RNAi experiments, it is critical to optimize transfection conditions for a given cell type. While the use of Lipofectamine 2000 has been described, certain cell types may not exhibit a high level of transfection efficiency using this reagent.
2. Some cell types may exhibit slower doubling times and may require longer periods of time to establish TGS following siRNA transfection. In these instances, waiting 48–72 h after siRNA transfection may provide a better window to assess silent chromatin marks using ChIP.
3. For ChIP experiments, optimization of chromatin shearing conditions is critical and must be determined separately for each cell type.
4. Some genes may not be amenable to siRNA-mediated TGS. Low levels of sense transcription across the target promoter region may be necessary for the siRNA antisense or guide strand to recognize and target. Additionally, highly euchromatic genes may not be susceptible to siRNA-directed heterochromatin formation.

Acknowledgments

This work was supported by a City of Hope Graduate School Internal Fellowship to D.H.K. and National Institutes of Health grants R37-AI29329 and R01-AI42552 to J.J.R.

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