Transcriptional Silencing by Hairpin RNAs Complementary to a Gene Promoter

Yongjun Chu, Roya Kalantari, David W. Dodd, and David R. Corey

Double-stranded RNAs can target gene promoters and inhibit transcription. To date, most research has focused on synthetic RNA duplexes. Transcriptional silencing by hairpin RNAs would facilitate a better understanding of endogenous RNA-mediated regulation of transcription within cells. Here we examine transcriptional silencing of progesterone receptor (PR) expression by hairpin RNAs. We identify the guide strand as the strand complementary to an antisense transcript at the PR promoter and that hairpin RNAs are active transcriptional silencing agents. The sequence of the hairpin loop affects activity, with the highest activity achieved when the loop has the potential for full complementarity to the antisense transcript target. Introduction of centrally mismatched bases relative to the target transcript does not prevent transcriptional silencing unless the mismatches are present on both the guide and passenger strands. These data demonstrate that hairpin RNAs can cause transcriptional silencing and offer insights into the mechanism of gene modulation by RNAs that target gene promoters.

Introduction

SMALL DUPLEX RNAs THAT ARE complementary to gene promoters can modulate gene expression (Janowski and Corey, 2010; Morris, 2011). In some cases promoter-targeted RNAs can act as transcriptional repressors, while in other cases they activate transcription. These RNAs do not appear to bind directly to chromosomal DNA. Instead, they can bind to sense or antisense noncoding transcripts that overlap the promoter regions of the target gene (Han et al., 2007; Schwartz et al., 2008). The action of promoter-targeted RNAs requires argonaute (AGO) protein (Kim et al., 2006; Janowski et al., 2006; Li et al., 2006; Chu et al., 2010). There are 4 AGO proteins in human cells (AGO1–4). Both AGO1 and AGO2 have been implicated in RNA-mediated regulation of transcription. The small RNAs recruit AGO to the noncoding transcript at the gene promoter.

MicroRNAs (miRNAs) are approximately 22 nucleotide RNAs that regulate processing of mRNA (Carthew and Sontheimer, 2009). In animals, most miRNAs are processed from longer transcripts by the ribonucleases droscha and dicer. Droscha acts to separate a partially paired hairpin from the parent transcript while dicer trims the droscha product to produce the mature miRNA. Until recently dicer was regarded as an indispensable component of the RNA interference (RNAi) machinery but it has been shown that maturation of some miRNAs depends on AGO 2 instead (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010; Betancur and Tomari,

2012). In these examples, rather than remove the RNA hairpin loop as dicer does, AGO2 cleaves between positions 10 and 11 within the hairpin stem.

To date, almost all studies of small RNAs that regulate gene transcription have used synthetic RNA duplexes consisting of 2 annealed single strands. Hairpin RNA, however, would better mimic the structure of natural microRNAs and be a better tool for investigating the potential for endogenous mechanisms of transcriptional silencing. Hairpin RNAs are the foundation for gene silencing by expressed RNAs, and a better understanding of their function would facilitate using viral vectors to express promoter targeted RNAs. There have been several reports of using virally expressed hairpin RNAs designed to act through dicer to target the promoter of human immunodeficiency virus (HIV)-1 replication (Suzuki et al., 2005; Suzuki et al., 2011; Weinberg et al., 2006). There have been no systematic studies investigating the effects of mutating key features of hairpins that target gene promoters on transcriptional gene silencing or investigating the role of dicer or AGO2 cleavage pathways.

Here we correlate the structure of promoter-targeted RNAs with their function and show that synthetic hairpin RNAs can be active as transcriptional silencing agents. We specifically focus on hairpin frameworks based on miR-451. Changing the sequence within the hairpin loop and within the central region of the stem affects activity and provides insights into the mechanism of gene inhibition.

Materials and Methods

Cell culture and transfection

T47D cells (American Type Culture Collection) were maintained in RPMI-1640 media supplemented with 10% (v/v) fetal bovine solution, 0.5% (w/v) nonessential amino acids, 0.4 units/mL bovine insulin (all reagents from Sigma). Cells were cultured at 37°C and 5% (v/v) CO₂. All RNAs were synthesized by Integrated DNA Technologies. Oligofectamine RNAiMAX (Invitrogen) was used as lipid for all RNA transfections.

A reverse transfecting method was used for all transient transfection experiments. The cells were first detached using trypsin and then seeded in 6-well plates (Costar), to which appropriate RNA duplex/lipid/OPTI-MEM mixture was added (final concentration 25 nM for each RNA). Media was changed 2 days later and the cells were harvested for 24 hours later for mRNA analysis and 48 hours later for protein analysis.

Western blotting

Western blots were performed on protein lysates (30 or 40 µg per sample). Primary antibodies included monoclonal anti-PR (3172, Cell Signaling) and anti-β-actin (internal control). Protein was visualized using secondary anti-mouse (Jackson Immunolabs) and Supersignal developing solution (Pierce).

RNA analysis

Expression of progesterone receptor (PR) and antisense PR transcript was evaluated by real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA from treated T47D cells was extracted using Trizol (Invitrogen). Samples of 2 µg were treated with DNase 1 (6355, Worthington) first, followed by reverse transcription using random primers (Applied Biosystems) with the High Capacity cDNA Archive kit (Applied Biosystems). Products were detected using TaqMan Gene Expression Assay (PGR, Applied Biosystems) with 50 ng of complementary DNA. Data were normalized relative to measured levels of GAPDH (Applied Biosystems). Error is expressed as standard deviation from the mean.

Results and Discussion

We had previously described evidence that an antisense transcript overlapping the PR promoter was the target for small RNAs that modulate PR expression (Schwartz et al., 2008; Yue et al., 2010). The PR gene has 2 transcription start sites that produce 2 different mRNAs. These mRNAs code for 2 isoform proteins, PR-A and PR-B. For studies of PR gene silencing with hairpin RNAs, it is essential that the identity of the guide strand be determined. To accomplish this, we introduced mutations into either strand of PR9, a duplex that inhibits transcription and spans the regions from -9 to +10 relative to the most upstream transcription start site of the PR promoter to create duplexes PR9.1 and PR9.2 (Fig. 1A).

We found that PR9.2, which contains mutations within the strand complementary to the antisense transcript, was not able to silence gene expression (Fig. 1B, D). By contrast, PR9.1 that contains mutations within the strand complementary to sense transcript was an efficient inhibitor of PR gene expression. As a positive control we evaluated PRC1 (Fig. 1A), a

duplex RNA complementary to PR mRNA that acts through standard post-transcriptional gene silencing and is a potent inhibitor of PR expression. A duplex containing 4 mismatches (M4) was used as a negative control.

We have previously used a battery of mismatched and scrambled control duplexes to establish that transcriptional regulation requires seed sequence complementarity to the PR promoter (Janowski et al., 2005). We further demonstrated specificity by showing that several different duplex RNAs silence gene expression, with the only commonality among them being complementarity to the PR promoter. We had previously used the nuclear run-on assay to show inhibition of transcription and chromatin immunoprecipitation to show decreased recruitment of RNA polymerase 2 (Janowski et al., 2006; Schwartz et al., 2008). These data suggest that the antisense transcript is the target strand for transcriptional silencing at the PR promoter and are consistent with previous results showing recruitment of AGO2 to the antisense transcript by PR9 (Schwartz et al., 2008; Chu et al., 2010).

We next tested hairpin RNAs with stem sequences analogous to PR9 or PRC1 to evaluate whether constraining the duplex RNA as a hairpin would affect gene silencing (Fig. 1C, D). The terminal 3' and 5' bases were taken from miR451. miR451 is an endogenously expressed miRNA that is of special interest because it is matured through cleavage by AGO2 rather than dicer (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). The sequences of the hairpin loops were chosen so that, upon processing and hybridization, the loop would be able to base-pair with the antisense transcript (for PR9) or the mRNA (for PRC1) target if the RNA was processed similarly to miR451.

We observed that hairpin HPR9 (Fig. 1A) was an active silencing agent (Fig. 1C, D). This result demonstrates that constraining a promoter-targeted RNA into a hairpin loop structure and placing it into a framework resembling Drosha-processed miR-451 does not interfere with transcriptional silencing. Transcriptional silencing by hairpin HPR9 was potent, with approximately 50% inhibition achieved at a 6 nM concentration (Fig. 1E).

We then examined whether modifying the loop regions to reduce complementarity to the target transcripts would affect activity of hairpin RNAs complementary to PR promoter transcript or PR mRNA (Fig. 2A). These loop mismatches fall outside the seed sequence (bases 2–8) where exact base-pairing is critical for optimal activity. The mutations were chosen because of their potential to form unusually stable hairpin loop structures (Cheong et al., 1990). For hairpins HPR9.1 and HPR9.2 targeting the promoter transcript (Fig. 2B), and HPRC1.1 and HPRC1.2 (Fig. 2A) complementary to PR mRNA (Fig. 2C), we found that introducing mismatches within the hairpin loop reduces gene silencing. These data suggest that complementarity within the hairpin loop contributes to efficient recognition of RNA targets and is consistent with processing of the passenger strand by AGO2 to form a mature guide strand.

Normally, post-transcriptional gene silencing by a fully complementary duplex involves AGO2-mediated cleavage of the target mRNA. To investigate whether cleavage of the promoter antisense transcript was necessary for transcriptional gene silencing, we mutated the hairpin RNA at position 10 to create a mismatch between the guide RNA and the antisense transcript (Fig. 3A). Mismatched bases at position 10

FIG 1. Inhibition of progesterone receptor (PR) expression by duplex and hairpin RNAs. **(A)** Duplex and hairpin sequences. **(B)** Real-time polymerase chain reaction (RT-qPCR) measurement of PR inhibition by duplex RNAs. **(C)** RT-qPCR measurement of PR mRNA inhibition by hairpin RNAs. **(D)** Western blot analysis showing inhibition of PR protein expression by duplex and hairpin RNAs. **(E)** Western blot analysis showing dose-dependent knockdown of PR by HPR9. Unless otherwise noted, the transient transfections were carried out in T47D cells using 25 nM RNA. Bases that are mismatched relative to pairing at the PR promoter are in boldface. PR mRNA levels are relative to treatment with MM4 negative control duplex.

are known to disrupt AGO2-mediated cleavage of RNA targets (Wang et al., 2008). We found that hairpin HPR9.3 containing the position 10 mismatch remained an effective agent for transcriptional silencing (Fig. 3B), leading us to conclude that transcript cleavage is not necessary for transcription silencing.

This finding is consistent with our earlier observation using RNA immunoprecipitation (RIP). We had observed that AGO2 is recruited to the antisense transcript even though the primers for RIP are on either side of the potential AGO2 cleavage site and RIP would not be able to detect cleaved product (Chu et al., 2010). It is likely that the antisense transcript acts as a docking platform to anchor the promoter-target RNA/AGO complex near the gene promoter and transcriptional regulatory apparatus and that cleavage of the noncoding transcript is unnecessary for RNA-mediated regulation of PR.

In contrast to the lack of effect on transcriptional silencing from the central mutation, the introduction of a mismatch at position 10 on PRC1 to create hairpin HPRC1.3 abolished activity, confirming the importance of AGO2-mediated cleavage for post-transcriptional gene silencing. These data suggests an important mechanistic distinction between post-transcriptional gene silencing and RNA-mediated transcriptional gene silencing.

As noted above, AGO2 processes miR-451 by cleaving the passenger strand between bases 10 and 11 within the hairpin stem (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010; Betancur and Tomari, 2012). To determine whether this might also occur during transcriptional silencing, we introduced a mismatched bulge into hairpin HPR9.4 that should not be a substrate for cleavage by AGO2 (Fig. 3A). Even though this duplex is capable of perfect pairing with the antisense transcript target, the mutation abolished activity (Fig.

FIG. 2. RT-qPCR analysis showing effect of mismatches within the hairpin loop on inhibition of PR expression. **(A)** Hairpin sequences. **(B)** Effect of mismatches within the loop of hairpins complementary to the PR promoter. **(C)** Effect of mismatches within the loop of a hairpin targeted to PR mRNA. Bases that are mismatched relative to sequences encoded by the PR promoter are in boldface. The guide strand relative to association with antisense transcript at the PR promoter is underlined. PR mRNA levels are relative to treatment with HM4 negative control duplex.

FIG. 3. RT-qPCR analysis examining the effect of altering potential argonaute cleavage sites on inhibition of PR expression. **(A)** Hairpin sequences. **(B)** Effect of a mismatch relative to target RNA at position 10 within the hairpin stem region. **(C)** Effect of introducing a mismatch within the stem region of the hairpin at positions 10 and 11. Bases that are mismatched relative to pairing at sequences encoded by PR promoter are in boldface. The guide strand relative to association with antisense transcript at the PR promoter is underlined. PR mRNA levels are relative to treatment with HM4 negative control duplex.

3C). This result is consistent with the hypothesis that AGO2 can process small hairpin RNAs during transcriptional silencing.

PR9.1 also contains a bulge at position 10, but in contrast to HPR9.4, it effectively silences PR expression. PR9.1 is a simple duplex and would be expected to act like a typical silencing RNA without the need for further processing. HPR9.4 is a hairpin that has been designed to incorporate features from miR-451, a miRNA known to be processed by AGO2. The consequences of introducing mismatches differ because the context of the sequences differs.

Our data demonstrate that synthetic hairpin RNAs can be effective agents for transcriptional silencing and suggest that they can be processed through a pathway that requires AGO2 activity. Maximal activity requires that both the hairpin stem and hairpin loop be complementary to the target transcript. A lack of complementarity within the hairpin stem may affect processing and appears to disrupt gene silencing. These data begin to define the use of hairpin RNAs as transcriptional silencing agents and demonstrate their value as tools for investigating mechanism.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dr. David R. Corey
Department of Pharmacology
University of Texas Southwestern Medical Center
Dallas, TX 75390

E-mail: david.corey@utsouthwestern.edu

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