

RESEARCH ARTICLE

Asymmetrically designed siRNAs and shRNAs enhance the strand specificity and efficacy in RNAi

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ABSTRACT

RNAi can mediate allele-specific silencing, and offers an attractive approach for treatment of human diseases caused by dominant, gain-of-function gene mutations. However, allele-specific silencing requires that the RNAi target the mutated region of the mRNA, limiting the choices of the target sequences. This often results in the use of a suboptimal siRNAs or shRNAs. Unfavorable strand asymmetry, which leads to the sense strand rather than the antisense strand to be incorporated into RNA-induced silencing complex (RISC), can cause poor RNAi efficacy. We systematically tested an approach that places mismatches at or near the 5' of the antisense strand to create favorable strand asymmetry. Here we show that this approach can enhance the RNAi efficacy of not only siRNAs but also shRNAs synthesized from genes, which can be placed in various viral vectors. Thus, this design of asymmetric shRNAs could be potentially used in silencing dominant, gain-of-function gene mutations for gene therapy.

KEYWORDS: Gene silencing, RNAi therapy, reverse genetics, ALS, neurodegenerative disease, SOD1

INTRODUCTION

RNAi is a conserved eukaryotic mechanism that is triggered by the presence of double stranded RNA (dsRNA) in cells. In RNAi, long dsRNA or hairpin RNA are processed by Dicer, an enzyme of the RNase III family, into 21-25 nucleotide double-stranded fragments, termed small interfering RNAs (siRNAs) (Zamore et al, 2000; Bernstein et al, 2001). The siRNAs interact with proteins Dicer and TRBP (R2D2 in *Drosophila*), which facilitate the formation of a siRNA/multi-protein complex called RISC (RNA-induced silencing complex) loading complex (RLC) (Tomari et al, 2004). The RLC then interacts with additional proteins including Ago2 to form the active RISC that contains one of the two siRNA strand

(called the guide strand). This RISC is capable of recognizing the target RNA by Watson-Crick base pairing with the guide strand and cleaves the target RNA, which is then released, and the RISC goes on to catalyze a new cycle of target recognition and cleavage (Tomari and Zamore, 2005).

RNAi can also be triggered by microRNAs (miRNAs), which are synthesized by RNA polymerase II and are embedded in long transcripts, called pri-miRNA (Bracht et al, 2004; Cai et al, 2004; Lee et al, 2004). Pri-miRNA is processed by RNase III enzyme Drosha and its partner Pasha to form pre-miRNA, which is ~70 nt long and folds into a hairpin structure (Lee et al, 2003; Denli et al, 2004.). It is then exported by Exportin 5 from the nucleus to the

cytoplasm (Yi et al, 2003; Bohnsack et al, 2004; Lund et al, 2004; Zeng and Cullen, 2004), where it is further processed to form single stranded miRNA (Grishok et al, 2001; Hutvagner et al, 2001; Jiang et al, 2005; Ketting et al, 2001; Forstemann et al, 2005). This processing step may be tightly coupled with loading the miRNA into the RISC, which is capable of either cleaving the target RNA (if the target perfectly complements the miRNA in sequence) or mediating translational silencing (if the miRNA contains mismatches to multiple sequences in the target RNA). This process has been mimicked by shRNAs synthesized from either Pol III or Pol II promoters (Xia et al, 2002; Shi, 2003; Zeng and Cullen, 2003; Zhou et al, 2005).

Because of its sequence specificity, RNAi has become a powerful tool in reverse genetics for investigation of gene function. In addition, it has been increasingly applied as a therapeutic strategy in cells, animals and even in humans (Uprichard, 2005). In these applications, RNAi is delivered as either synthetic siRNAs or gene-based synthesis of shRNAs that mimics pre-miRNAs in structure and function. However, silencing efficacy of many siRNAs is variable (Khvorova et al, 2003; Hsieh et al, 2004; Reynolds et al, 2004) and the specificity of RNAi is imperfect (Jackson et al, 2003). In research applications these are not serious problems because one can screen for effective siRNAs and avoid the ones with poor efficacy or specificity. However, in therapeutic applications this may be problematic. For example, in situations where silencing a mutant allele is therapeutic but silencing a wild type gene may lead to serious toxicity, selective silencing of the mutant allele will be required. To accomplish this siRNAs targeting the mutation site must be used (Abdelgany et al, 2003; Ding et al, 2003; Gonzalez-Alegre et al, 2003; Miller et al, 2003; Miller et al, 2004). Consequently one may be forced to select siRNAs within a limited repertoire of sequences.

Several hypotheses have been proposed for poor siRNA efficacy. Studies have demonstrated that factors such as high GC content of siRNA (Amarzguioui et al, 2004; Chalk et al, 2004; Reynolds et al, 2004; Holen et al, 2005), the inaccessibility of the target region (Holen et al, 2002; Brown et al, 2005; Heale et al, 2005), and the unfavorable strand asymmetry of the siRNA (Khvorova et al, 2003; Schwarz et al, 2003) could lead to lower function of RNAi. While other theories remain disputable, the asymmetry rule in RNAi seems well-accepted and is perhaps the most dominant factor. The strand asymmetry is defined as follows: For each siRNA, only one of the two strands, the guide strand, will be loaded into the RISC and execute RNAi. The other strand, called passenger strand, will be destroyed. The thermodynamic stability of base pairing at the two ends of the siRNA predicts the likelihood of which strand will become the guide or the passenger strand. The strand with its 5' base pairing less stable than its 3' base pairing is more probable to enter RISC and vice versa. If the base pairing at the two ends has similar stability, then both strands may enter RISC with similar probabilities and mediate RNAi with similar potencies. Therefore, those siRNAs with stability of their end base pairing favoring the sense strands (as opposed to the antisense strand that is

complementary to the intended target) to enter the RISC will have poor RNAi efficacy, thus having unfavorable asymmetry.

Strand asymmetry can also affect RNAi specificity. RNAi can silence unintended targets, albeit to a lesser degree than the intended one. This is called off-target effects (Jackson et al, 2003). Because the critical binding energy of the RISC to the target RNA resides in the 5' half of the guide strand (Haley and Zamore, 2004), homology between this region of the guide strand and other unintended RNAs can lead to off-target silencing (Jackson et al, 2003). This implies that, if both strands of the siRNA can enter the RISC, the probability of off-target silencing will increase. Thus, by programming the asymmetry into the design of siRNA so that only the selected strand enters the RISC, the probability of off-target effects is likely to decrease.

Previous siRNAs design strategies have focused on target regions where natural asymmetric siRNAs can be found (Reynolds et al, 2004; Chalk et al, 2005). This approach may not be helpful in situations where the target region is confined (e.g., where RNAi is need for silencing the expression of a mutated gene specifically), and within this confined region no favorably asymmetric siRNAs can be found. In these situations, weakening base pairing by incorporation of mismatches at the 5' of the intended guide strand can create strand asymmetry favoring the intended guide strand (Holen et al, 2002; Schwarz et al, 2003; Holen et al, 2005; Uprichard, 2005; Schwarz et al, 2006). In this study, we tested the effectiveness of this design strategy in converting siRNAs with unfavorable asymmetry to the ones with favorable asymmetry in human cells. We show that this strategy can effectively convert a strand of siRNA that is originally favored to become the passenger strand to the one that is favored to become the guide strand and vice versa, and this conversion enhances RNAi efficacy. Furthermore, we show that this strategy can be incorporated into the design of shRNA and enhance its RNAi efficacy and strand specificity.

MATERIALS AND METHODS

siRNA and shRNA preparation

Single stranded RNAs were purchased from Dharmacon Research, deprotected according to the manufacturer's instructions, and annealed as described previously (Ding et al, 2003). To construct the shRNA vectors, the two strands of synthetic DNA oligonucleotides were annealed, and subcloned into a RNA polymerase III promoter (U6) driven vector using the restriction sites *Pme* I and *Pst* I. The parent U6 vector was generated by cloning the U6 promoter (-315 to +1) from pmU6 plasmid into Bluescript (Sui et al, 2002). The DNA strands contained 19 or 21 nt sense and antisense strands (that matches the target sequences) linked by a 9-nucleotide loop (UUCAAGAGA). The sense strand terminates with 5 consecutive Thymidines (TTTTT). All constructs were verified by nucleotide sequencing.

Cell culture and transfection

Human embryonic kidney 293 cells were maintained in Dulbecco's Modified Eagle Media (Invitrogen)

supplemented with 10% (v/v) FBS plus 100 U/ml penicillin and 100 µg/ml streptomycin. Twenty-four hours before experiments, cells were detached with trypsin-EDTA (0.05% (w/v) Trypsin, 0.53mM EDTA·4Na) at 70-90% confluency, and transferred into the wells of appropriate plates at 30% cell density. Transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions on the following day. For testing siRNAs, the following constructs and concentrations were used in transfection: The firefly luciferase (pGL2 control vector, Promega) with target sequence inserted into the 3' UTR, 2.0 µg/ml; Renilla luciferase vector (pRL-TK, Promega), 0.1 µg/ml; and siRNA, 0.002 to 31.25 nM. For testing shRNA-expressing constructs, the following constructs and concentrations were used in transfection: The target firefly luciferase, 1.0 µg/ml; Renilla luciferase vector (pRL-TK, Promega), 0.1 µg/ml, and shRNA-expression vector, 0.5 µg/ml.

Dual luciferase assay

A modified dual luciferase system (Promega) was used to quantify RNAi efficiency in cell culture. To generate specific luciferase target vectors for this study, a synthetic double-stranded oligonucleotide containing the restriction sites *Nde* I and *Spe* I was first engineered into the 3' UTR of the firefly luciferase vector (pGL2 control vector, Promega) at the PflMI site. Subsequently, a 39 nt fragment of human Cu Zn superoxide dismutase (*sod1*) gene (sense strand 5'-aggcatgttgagactgggcaatgtgactgtgacaaa-3', antisense strand 5'-ttgtcagcagtcacattgcccgaagtctccaacatgcct-3') was synthesized, annealed and cloned into the 3' UTR region of firefly luciferase vector either as natural sense-to-antisense (sense target) or reversed antisense-to-sense (antisense target) oligonucleotide duplex using the *Nde* I and *Spe* I sites. The modified firefly luciferase vectors express luciferase, but are sensitive to the cleavage of inserted *sod1* fragment by RNAi which causes the loss of poly A thus the degradation of luciferase mRNA. For testing RNAi efficacy, either sense-target or antisense-target vector was co-transfected with Renilla luciferase vector (pRL-TK, Promega) plus siRNA or the shRNA-synthesizing vectors into HEK293 cells in 96-well plates in quadruplicate using Lipofectamine 2000 reagent (Invitrogen). Twenty four hours after the transfection, cells were lysed with 20 µl Passive Lysis Buffer (Promega). Ten µl lysate from each well was transfer into a well in a strip (Thermo labsystems) and measured with a Veritas microplate luminometer (Turner Biosystem). The luminescence intensity ratio (Firefly/Renilla luciferase) was used for measuring the RNAi efficacy.

Northern blotting

One µg of each of the shRNAs was transfected into HEK293 cells in 6-well plates. Cells were harvested 24 hr post-transfection and the total RNA was extracted with Tri Reagent (Molecular Research Center). Ten µg of total RNA was loaded onto a mini 15% (w/v) denaturing polyacrylamide gel. The separated RNAs were transferred onto a BrightStar-Plus nylon membrane (Ambion) and cross-linked with UV. ³²P-labeled sense or antisense 21 nt synthetic RNAs were used as probes for detecting their complementary RNA strands. The radioactive RNA bands

were read with Fuji Phosphor Imaging system FLA-5000 (Fuji Medical Systems).

RESULTS AND DISCUSSION

Designed asymmetry switches strand preference and enhances the RNAi efficacy of the desired strand

Natural siRNAs (with perfectly complementary double strands) often have unfavorable strand asymmetry. For example, of the three siRNAs that we used previously against human Cu, Zn superoxide dismutase (*sod1*) gene (Ding et al, 2003), one (P10) silenced the sense strand target (ss-target), the *sod1* mRNA, better than the antisense strand target (as-target), thus having a strand specificity favoring ss-target; the other two (P9, P11) actually silenced the as-target better than the ss-target, indicating an unfavorable strand specificity toward as-target (Figure 1). As expected, the ones that preferentially silenced the as-target silenced the ss-target relatively inefficiently, with the half-inhibitory concentration (IC₅₀) against the ss-target at ~2nM for the P9 and 5nM for the P11, compared with those against the as-target at 0.07nM and 0.1nM, respectively. Thus, P9 and P11 were poor candidates of natural siRNAs.

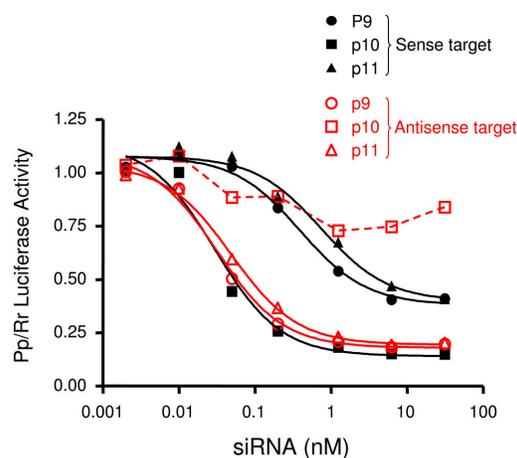


Figure 1. Asymmetric siRNAs. P9, P10 and P11 are 21nt siRNAs targeting the same region of human *sod1* mRNA. The sequence of P11 is shown in Fig. 2A. P9 and P10 target the *sod1* sequences 2- and 1-nt shifted toward the 5' of the *sod1* mRNA, respectively 28. Even though the three siRNAs target sequences only 1nt shifted from each other, their strand preference are very different, with P9 and P11 favoring the anti-sense strand target and P10 favoring the sense strand target. All data points are normalized to the luciferase activity in cells transfected with luciferase constructs but without siRNA and means of four experiments. Standard deviations are in the range of 1.4%-19% of the means and are omitted from the figure for clarity.

To determine whether the inefficient siRNAs could be converted into efficient ones, we took one of the inefficient siRNAs, P11 (Figure 2A), as a test model. Based on the asymmetry rule, we destabilized the base pairing at the 5' of the anti-sense strand of the siRNA (as-siRNA) by placing mismatches or an A:U pair at that end

(Figure 2A, S1-S3). Although P11 naturally favored the as-target (Figure 2B, see P11), destabilizing the base pairing at the 5' of the as-siRNA converted it to favoring sense target inhibition (Figure 2B, S1-S3). Conversely, destabilizing base pairing at the 5' of the sense strand of the siRNA (ss-siRNA) accentuated the preference to silencing the as-target (Figure 2B, A1-A3). Furthermore, destabilizing base pairing by replacing G:C with I:C at one end or the other similarly switched strand preferences (Figure 2B, IA, IS). If G:C at both ends are converted to I:C, the strand preference returned to the pattern of the P11 (Figure 2B, IS/IA). These changes are consistent with the predictions by the asymmetry rule.

In order to characterize the effects of the designed asymmetry quantitatively, we transfected the ss- and as-targets with different doses of siRNAs. The original P11 silenced the as-target maximally by 81%, with the IC₅₀ at 0.1nM. In contrast, it silenced the sense target maximally by only 60%, with IC₅₀ at 5nM (Figure 3, P11). By destabilizing the base pairing at the 5' end of the as-siRNA, the siRNA silenced the as-target maximally by only 56%, with an atypical dose-response curve that reached the maximal silencing at 2nM of siRNA and poorer silencing at the higher concentrations; in contrast, this siRNA silenced the sense target maximally by 79%, with the IC₅₀ at ~0.5nM (Figure 3, S2), a considerable improvement compared with the original P11. Thus, compared with the original P11, weakening the base pairing at the 5' of the as-siRNA reduced the RNAi efficiency against the as-target and enhanced the RNAi efficiency against the ss-target. Conversely, weakening the base pairing at the other end produced the reverse effect, enhancing the maximal silencing of the as-target while preventing silencing of the sense target completely (Figure 3, A2). If base pairing was weakened at both ends of the siRNA, the silencing pattern of the target reverted to the original P11 (Figure 3, S2/A2). These results support the predictions by the asymmetry rule and indicate that the asymmetry rule can be applied to increase the repertoire of siRNA targeting sites.

Application of the asymmetry rule in shRNAs enhances strand specificity and efficacy

shRNAs with either a 19 or 21 nt stem are commonly used in the literature. To discover the optimal strategy to incorporate the asymmetry rule in the design of shRNA, we first tested shRNAs with 19 nt stems with a mismatch placed within the first four nucleotides of either end of the shRNA stem (Figure 4A). In order to eliminate the effects of mismatches, we used the targets that perfectly match the siRNA strand under the test. The shRNA with two strands of the stem perfectly matched shows symmetrical silencing efficacy (Figure 4B, P11-19). Mismatches at positions 1 and 2 from the 5' of the sense strand (Figure 4A, A1-19, A2-19) enhanced the silencing of the as-target while mildly weakened the silencing of the ss-target (Figure 4B, A1-19, A2-19), as predicted by the asymmetry rule. However, mismatches at positions 3 and 4 from the 5' of the sense strand (Figure 4A, A3-19, A4-19) enhanced the silencing efficacy against the ss-target and did not change the silencing efficacy of the as-target (Figure 4B, A3-19, A4-19), suggesting that mismatches in these two

positions of the shRNA with 19 nt stem do not follow the asymmetry rule.

A mismatch at position 1 from the 5' end of the as-shRNA stem (Figure 4A, S1-19) also did not conform to the asymmetry rule. While it did not change the silencing efficiency of the as-target, it actually compromised silencing of the ss-target (Fig. 4B, S1-19) – contrary to what was expected by the asymmetry rule. Mismatches at positions 2 and 3 from the 5' of the as shRNA stem (Figure 4A, S2-19, S3-19) did not enhance the silencing efficacy against the ss-target, though the silencing of the as-target was diminished (Figure 4B, S2-19, S3-19). A mismatch at position 4 from the 5' end of the as shRNA stem (Figure 4A, S4-19) did not affect the silencing efficacy against either the ss- or as-target (Figure 4B, S4-19). Overall, most of the mismatches created at either end of the stem poorly conformed to the asymmetry rule in their silencing efficacy.

shRNAs mimic pre-miRNAs in their structure, processing and function. Most pre-miRNAs have stems longer than 21-nt in length (Griffiths-Jones, 2004). From these stems miRNA duplexes, including those that are asymmetric, are produced (Kim, 2005). Thus, shRNAs with stems 21-nt or longer might be processed better after incorporation of the asymmetry rule. To test this we designed shRNAs with 21-nt stems and with weakened base pairing (mismatches) at each of the positions 1-4 at both ends of the stem (Figure 5A). We cotransfected each of these plasmids that synthesize the shRNAs with the plasmids that synthesize the sense or antisense targets and determined their RNAi efficacy.

The P11 shRNA had similar strand preference as the P11 siRNA. It silenced the as-target better than the ss-target (Figure 5B, P11-21). When the mismatched base pairs were placed at the 5' of the ss-shRNA, the strand preference to the as-target was accentuated at positions 1 and 2 (Figure 5B, A1-21, A2-21), but reduced at position 3 and 4 (Figure 5B, A3-21, A4-21). Conversely, when the mismatches were placed at the 5' of the as-shRNA at positions 1-4, the strand preference is reversed from the original P11-21; the shRNA silenced the ss-target better than the as-target (Figure 5B, S1-21 to S4-21), although at the position 4, the strand preference diminished. We conclude that the best weak base pairing position for generating favorable strand preference is at position 2 of the sense strand (A2-favoring antisense target) and position 3 of the antisense strand (S3-favoring sense target), because mismatches at these positions generated the largest degree of strand asymmetry.

Previous studies using *Drosophila* embryo extract demonstrated that the fate of the two strands in siRNA is different during RISC assembly. R2D2 acts as a sensor for the asymmetry of the siRNA duplexes and binds to the thermodynamically stable end. Dicer then binds the other end that is less stable in its base pairing (Tomari et al, 2004). This results in the favored strand being incorporated into RISC and mediates RNAi, and the opposite strand being destroyed (Schwarz et al, 2003; Matranga et al, 2005). The switch of the RNAi efficacy of

<p>SENSE TARGET 5' -CAUGUUGGAGACUUGGGCAAUGUGACUGCUCA-3'</p> <p>ANTISENSE TARGET 3' -GUACAACCCUCUGAACCCGUACACUGACGAGU-5'</p>	<p>S1 5' -GAGACUUGGGCAAUGUGAAdTdT-3' *****</p> <p>3' -dTdTTCUCUGAACCCGUUACACUG-5'</p> <p>S2 5' -GAGACUUGGGCAAUGUGACdTdT-3' *****</p> <p>3' -dTdTTCUCUGAACCCGUUACACUU-5'</p> <p>S3 5' -GAGACUUGGGCAAUGUGAAdTdT-3' *****</p> <p>3' -dTdTTCUCUGAACCCGUUACACUU-5'</p>
<p>P11 5' -GAGACUUGGGCAAUGUGACdTdT-3' *****</p> <p>3' -dTdTTCUCUGAACCCGUUACACUG-5'</p>	<p>S1 5' -GAGACUUGGGCAAUGUGAAdTdT-3' *****</p> <p>3' -dTdTTCUCUGAACCCGUUACACUU-5'</p> <p>S3 5' -GAGACUUGGGCAAUGUGAAdTdT-3' *****</p> <p>3' -dTdTTCUCUGAACCCGUUACACUU-5'</p>
<p>A1 5' -GAGACUUGGGCAAUGUGACdTdT-3' *****</p> <p>3' -dTdTAUCUGAACCCGUUACACUG-5'</p> <p>A2 5' -UAGACUUGGGCAAUGUGACdTdT-3' *****</p> <p>3' -dTdTTCUCUGAACCCGUUACACUG-5'</p> <p>A3 5' -UAGACUUGGGCAAUGUGACdTdT-3' *****</p> <p>3' -dTdTAUCUGAACCCGUUACACUG-5'</p>	<p>IA 5' -IAGACUUGGGCAAUGUGACdTdT-3' *****</p> <p>3' -dTdTTCUCUGAACCCGUUACACUG-5'</p> <p>IS 5' -GAGACUUGGGCAAUGUGACdTdT-3' *****</p> <p>3' -dTdTTCUCUGAACCCGUUACACUI-5'</p> <p>IAIS 5' -IAGACUUGGGCAAUGUGACdTdT-3' *****</p> <p>3' -dTdTTCUCUGAACCCGUUACACUI-5'</p>

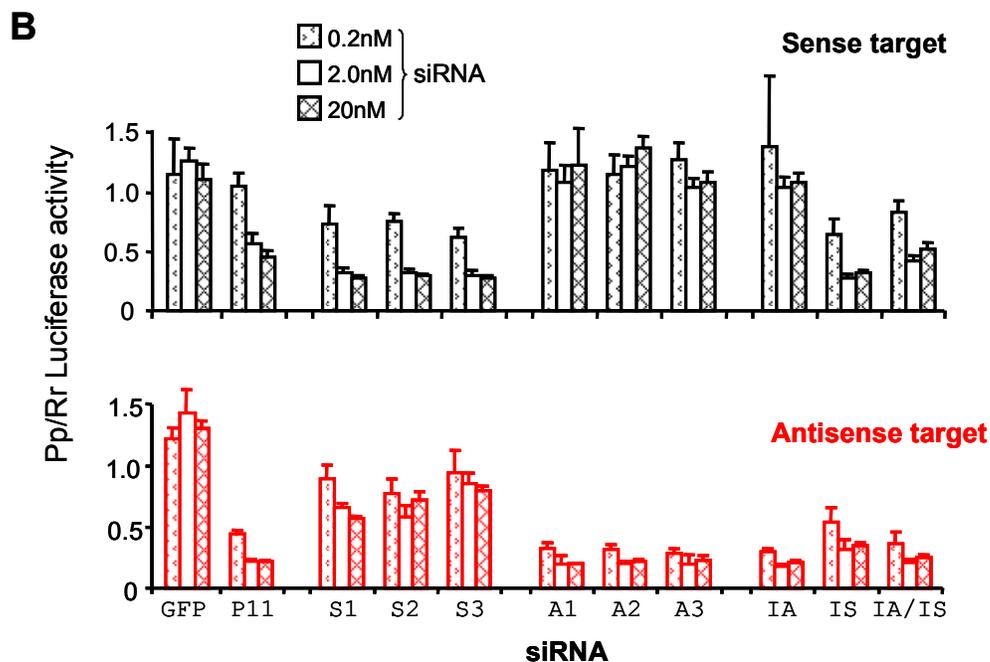


Figure 2. Natural strand preference can be designed by placing mismatches at the 5' end of the siRNA strand desired to be the preferred strand to go into the RISC. **(A)** Sense and antisense target sequences that were inserted into the 3' UTR (top left), P11 siRNA and its variations with mismatches placed at either end of the siRNA. **(B)** Silencing efficacy of various siRNAs. Notice that the sense strand of siRNA and antisense strand of the target are coded red and the antisense strand of siRNA and the sense strand of the target are coded black. All data values were normalized with the target transfection without siRNA. Each bar represents the average from 4 experiments. Error bars represent standard deviations.

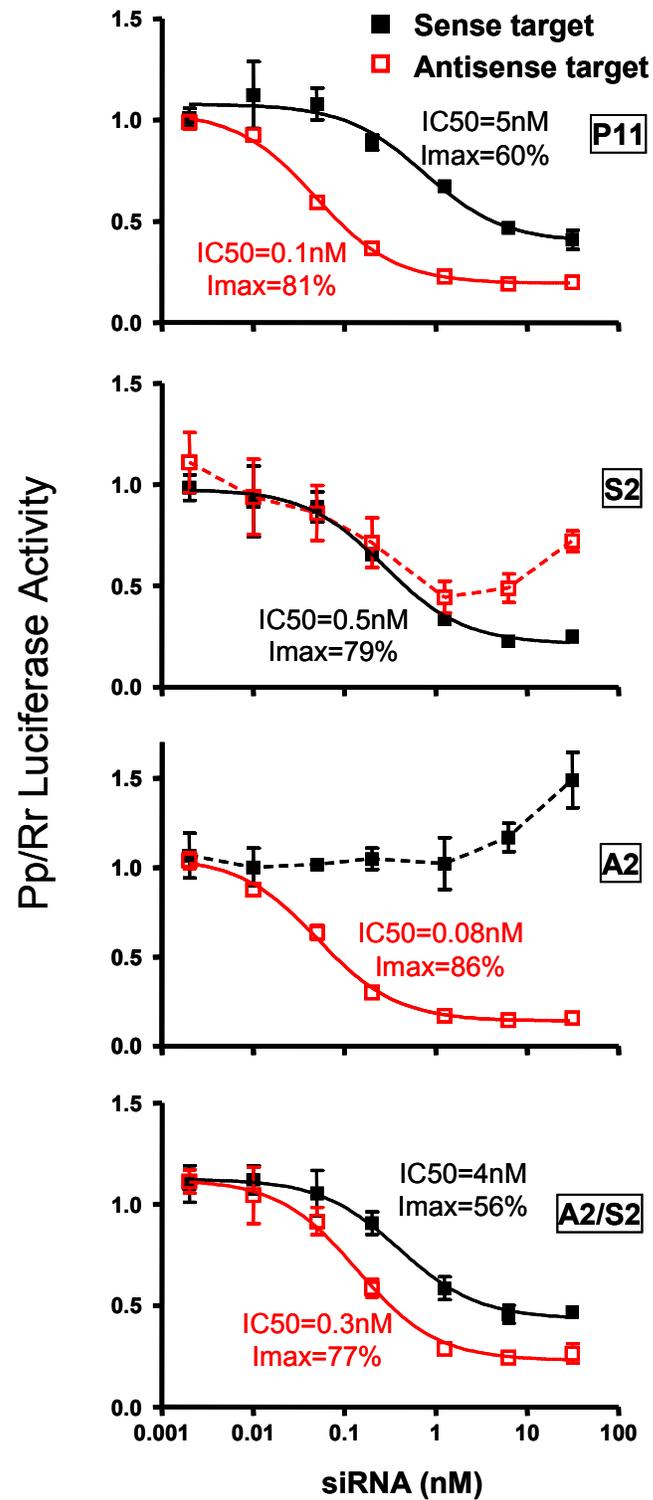


Figure 3. Designed asymmetric siRNA improves the RNAi efficacy of the desired strand and decreases the RNAi efficacy of the undesired strand. The sequences of P11, S2 and A2 siRNAs are shown in Figure 2A. The siRNA A2/S2 were generated by annealing the sense strand of A2 siRNA with the antisense strand of the S2 siRNA (see Figure 2A). All data points were normalized as described in Figure 1.

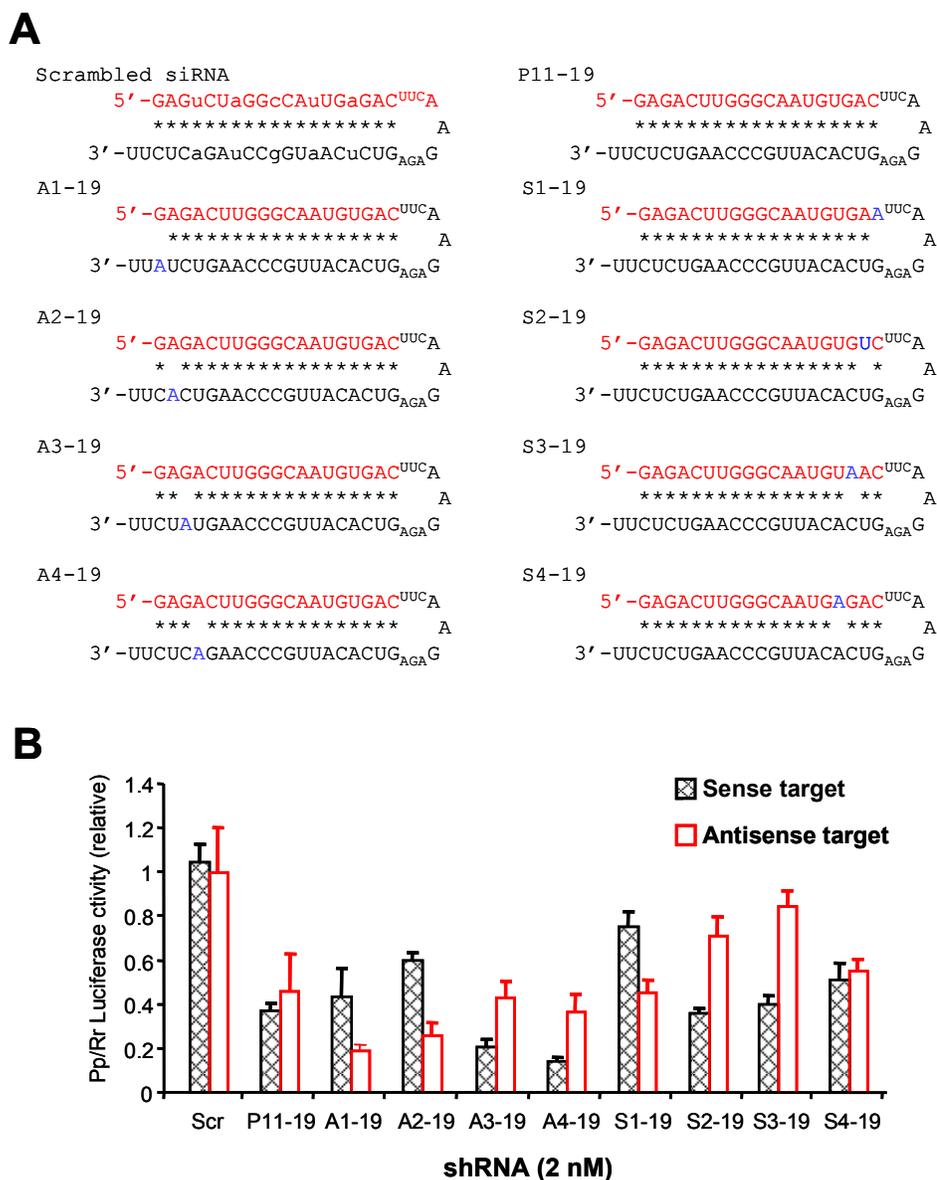


Figure 4. The strand preference of shRNAs with 19nt stem are not predicted by the asymmetry rule. **(A)** Sequences of shRNAs with 19 nt stem. Mismatches were placed at the first 4 positions of either strand of the stems. **(B)** The silencing efficacy of sense and antisense target by the dual luciferase assay. All the targets, including both the sense and antisense strands, perfectly complement their respective siRNA strands.

the two strands depending on the strand asymmetry in the shRNAs suggested that this might also be the case in mammalian cells (Figure 6A). To experimentally test this we performed northern blot analysis on RNA extracted from cells transfected with the shRNA-expressing vectors. We could detect the shRNAs produced by all three constructs (Figure 6B). The shRNAs were processed to siRNA. Both strands of siRNA from the P11 construct were detectable. However, only the favored strand could be detected from the S3 and A2 constructs (Figure 6B). This result is consistent with those obtained using the *Drosophila* embryo extracts (Tomari et al, 2004). The

sensor for shRNA asymmetry is not known yet. However, a recent experiment demonstrated that in *Drosophila*, Louquacious (Loqs) binds to miRNA and this binding is required for Dicer-1 processing of miRNAs (Forstemann et al, 2005). In humans, a Loqs homolog TRBP performs the similar roles (Chendrimada et al, 2005). Both Loqs and TRBP are dsRNA binding proteins similar to R2D2, and thus, could act as an asymmetry sensor for miRNAs or shRNAs.

Can the strategy of placing mismatches at position 2 of the sense strand or position 3 of the antisense strand of shRNA

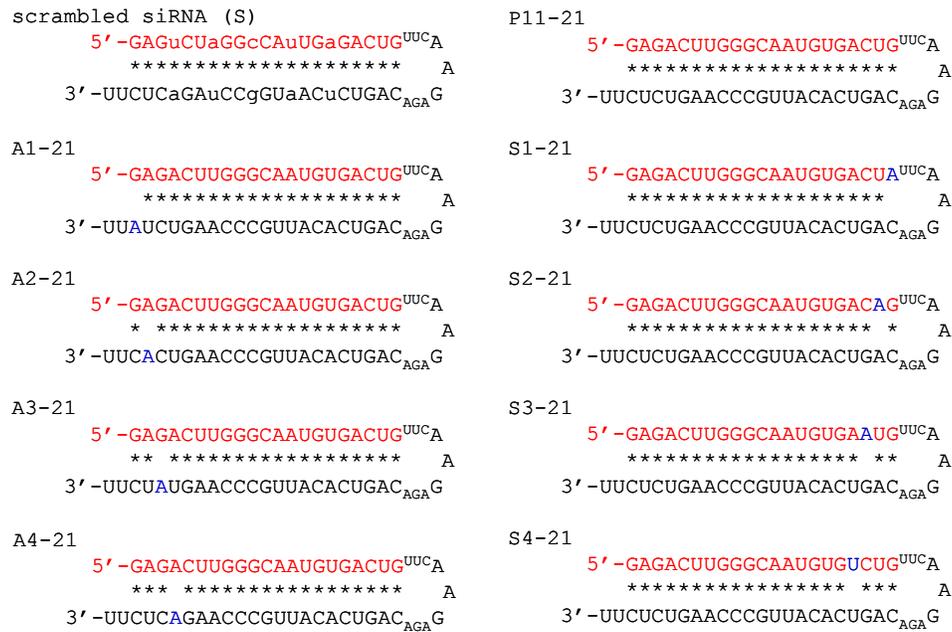
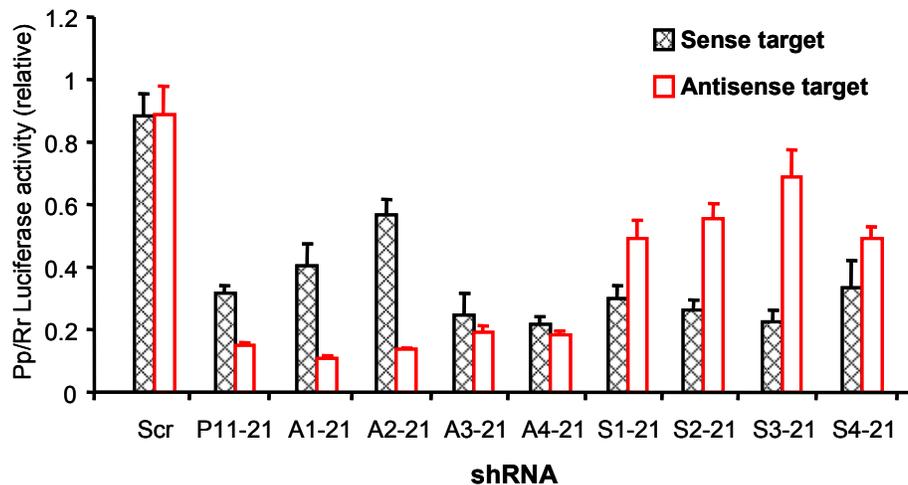
A**B**

Figure 5. The strand preference of shRNAs with 21nt stem are well predicted by the asymmetry rule. **(A)** Sequences of shRNAs with 21 nt stem. Mismatches were placed at the first 4 positions of either strand of the stems. **(B)** The silencing efficacy of the sense and antisense target were assayed by the dual luciferase assay. All the targets, including both the sense and antisense strands, perfectly complement their respective siRNA strands.

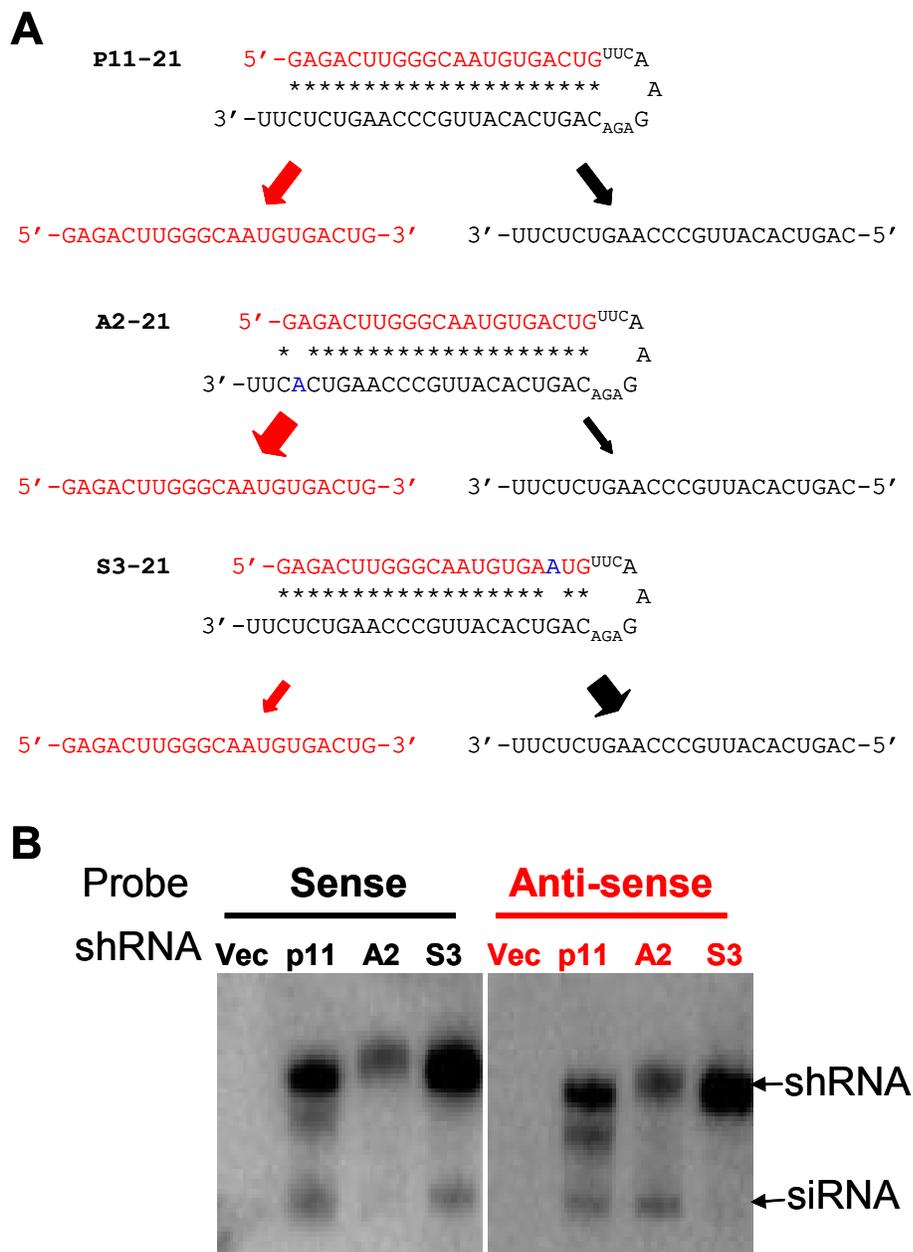


Figure 6. Designed asymmetric shRNAs were processed as predicted by the asymmetry rule. **(A)** Schematic processing of shRNA as predicted by the asymmetry rule. The thickness of the arrows indicates the degree of preference of that siRNA strand being incorporated into the RISC and thus being preserved. **(B)** Northern blot detecting shRNA and processed siRNA strands. In each lane, total RNA (10 μ g) from HEK293 cells transfected with the indicated shRNA constructs was loaded. The blots were detected using either the sense or the antisense RNA probes.

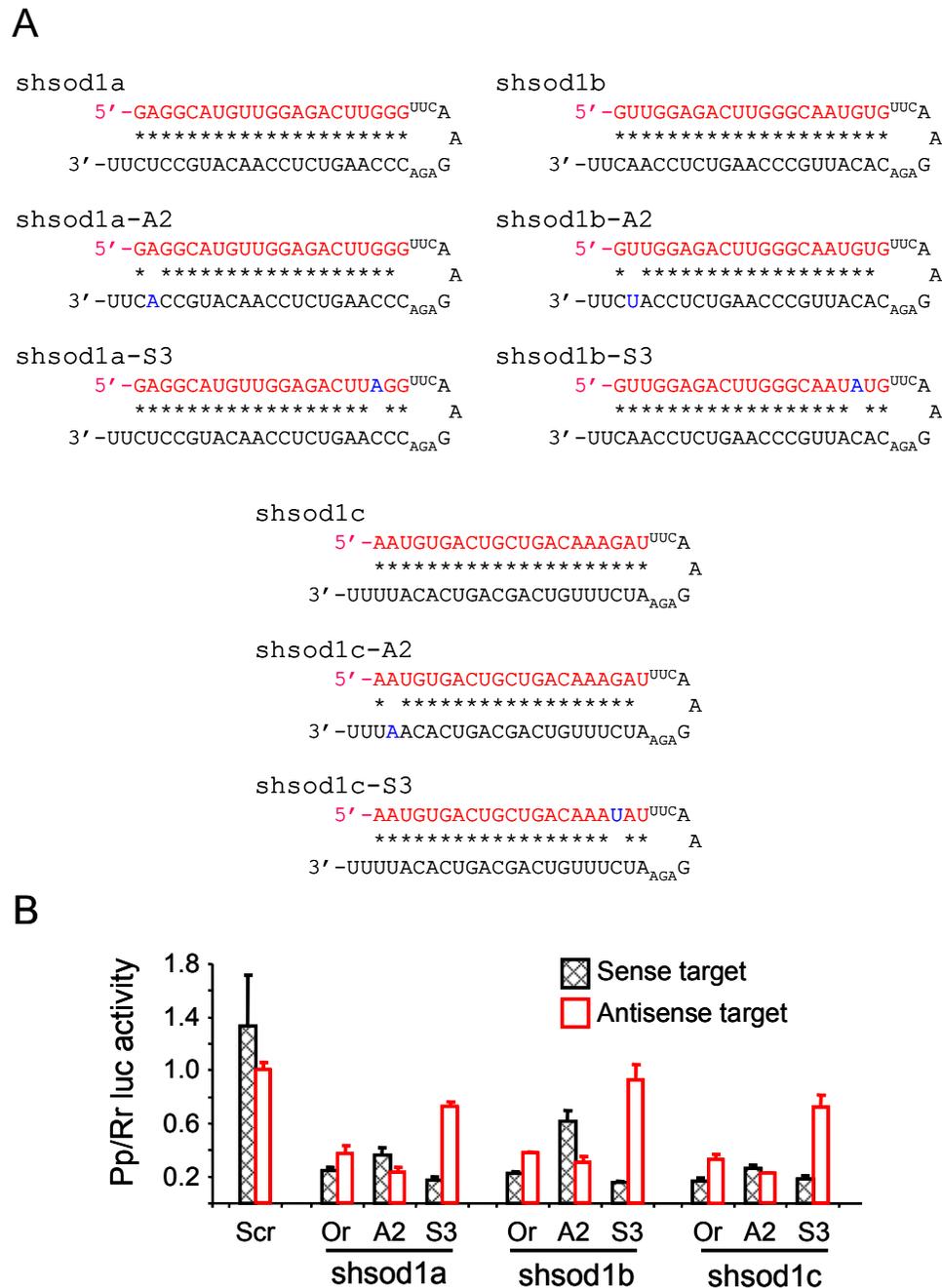


Figure 7. A mismatch at the 3rd position of the strand distal to the loop optimally produces shRNAs with favorable strand preference. Three additional sets of shRNAs with no mismatches, mismatches placed at A2 or S3 positions (**A**) were tested for their silencing efficacy to either the sense or the antisense targets (**B**). All the targets, including both the sense and antisense strands, perfectly complement their respective siRNA strands.

generate favorable strand preference in other shRNAs? To answer this question, we constructed three additional shRNAs and placed mismatches at the A2 and S3 positions (see Figure 7A). The original shRNAs silenced the sense strand slightly better than the antisense strand (Figure 7B, Or). Placing a mismatch at position A2 increased the silencing efficacy against the as-target and decreased the silencing efficacy against the ss-target for all three shRNAs, although the magnitude of these changes were small for two of the shRNAs, shsod1a and shsod1c

(Figure 7B, A2). On the other hand, placing a mismatch at S3 increased the silencing efficacy against the ss-target and decreased the silencing efficacy against the as-target (Figure 7B, S3). Based on these results, we conclude that a mismatch placed at S3 position most consistently enhances the strand specificity and RNAi efficacy. This design of asymmetric shRNAs could be used to generate effective shRNA viral vectors in silencing dominant, gain-of-function gene mutations for gene therapy.

CONCLUSIONS

- Weakening the base pairing at the end of naturally symmetric siRNA effectively converts the siRNA into asymmetric siRNA, which enhances not only the strand specificity but also the efficacy of siRNA.
- The incorporation of mismatch in the design of shRNA with 21 nt stem conforms to the asymmetry rule while the same strategy is not applicable to shRNA with 19 nt stem. Unlike siRNA, shRNA demonstrates the best asymmetry when a mismatch is placed at the position 2 of antisense strand or position 3 of sense strand instead of position 1.
- Asymmetrically designed siRNAs and shRNAs improve the gene silencing of intended targets and minimize the off-target effect, thus could be used in gene therapy of diseases caused by dominant, gain-of-function gene mutations.

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COMPETING INTERESTS

None declared.

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