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(54) **METHODS AND COMPOSITIONS FOR ENHANCING THE EFFICACY AND SPECIFICITY OF SINGLE AND DOUBLE BLUNT-ENDED SIRNA**

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(57) **ABSTRACT**

The present invention provides methods of enhancing the efficacy and specificity of RNAi using single or double blunt-ended siRNA. The invention also provides single and double-blunt ended siRNA compositions, vectors, and transgenes containing the same for mediating silencing of a target gene. Therapeutic methods are also featured.

Fig. 1

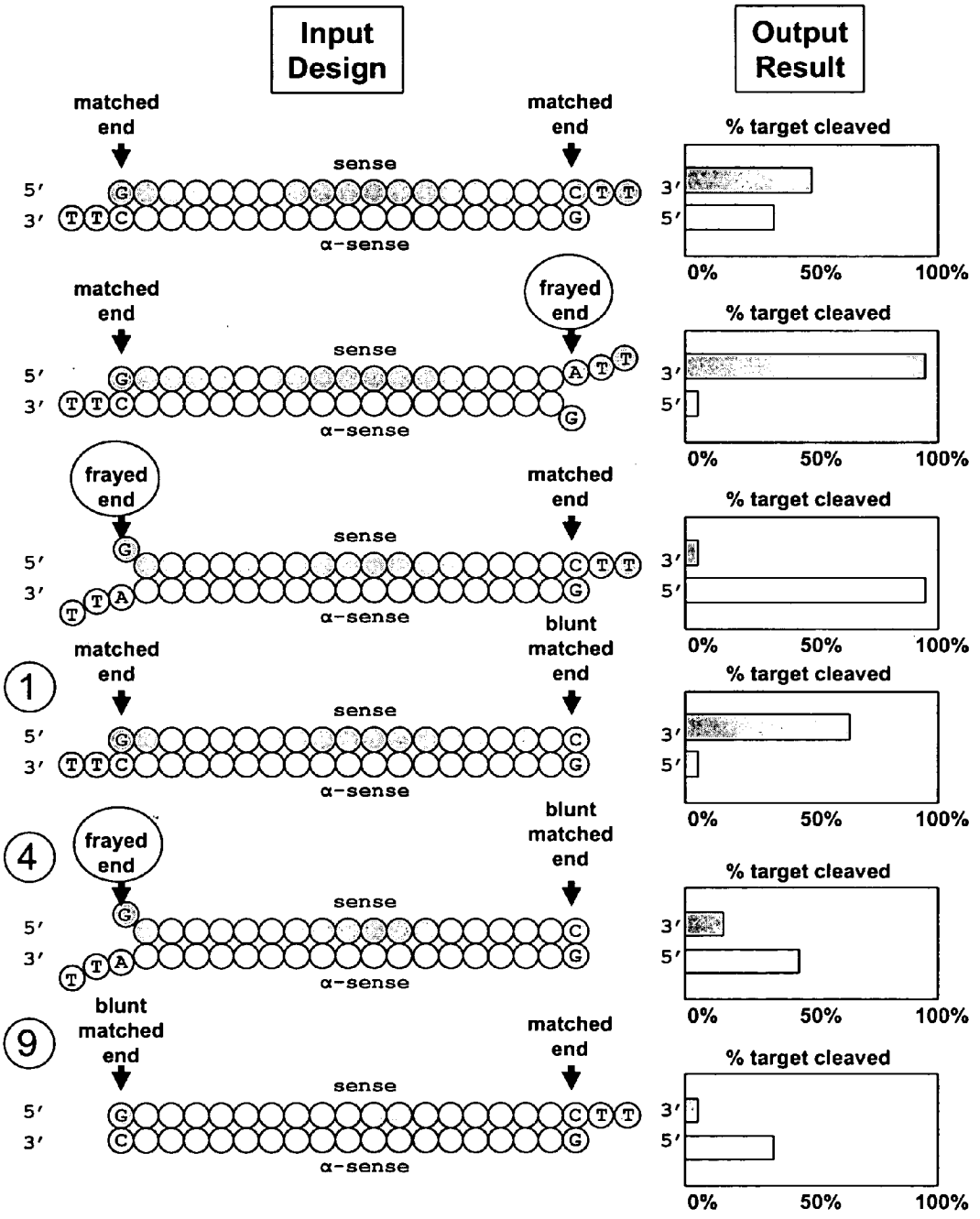

Input Design
Output Result
matched end
matched end
sense
5′
3′
α-sense
% target cleaved
0%
50%
100%
frayed end
blunt matched end
1
4
9

Fig. 2

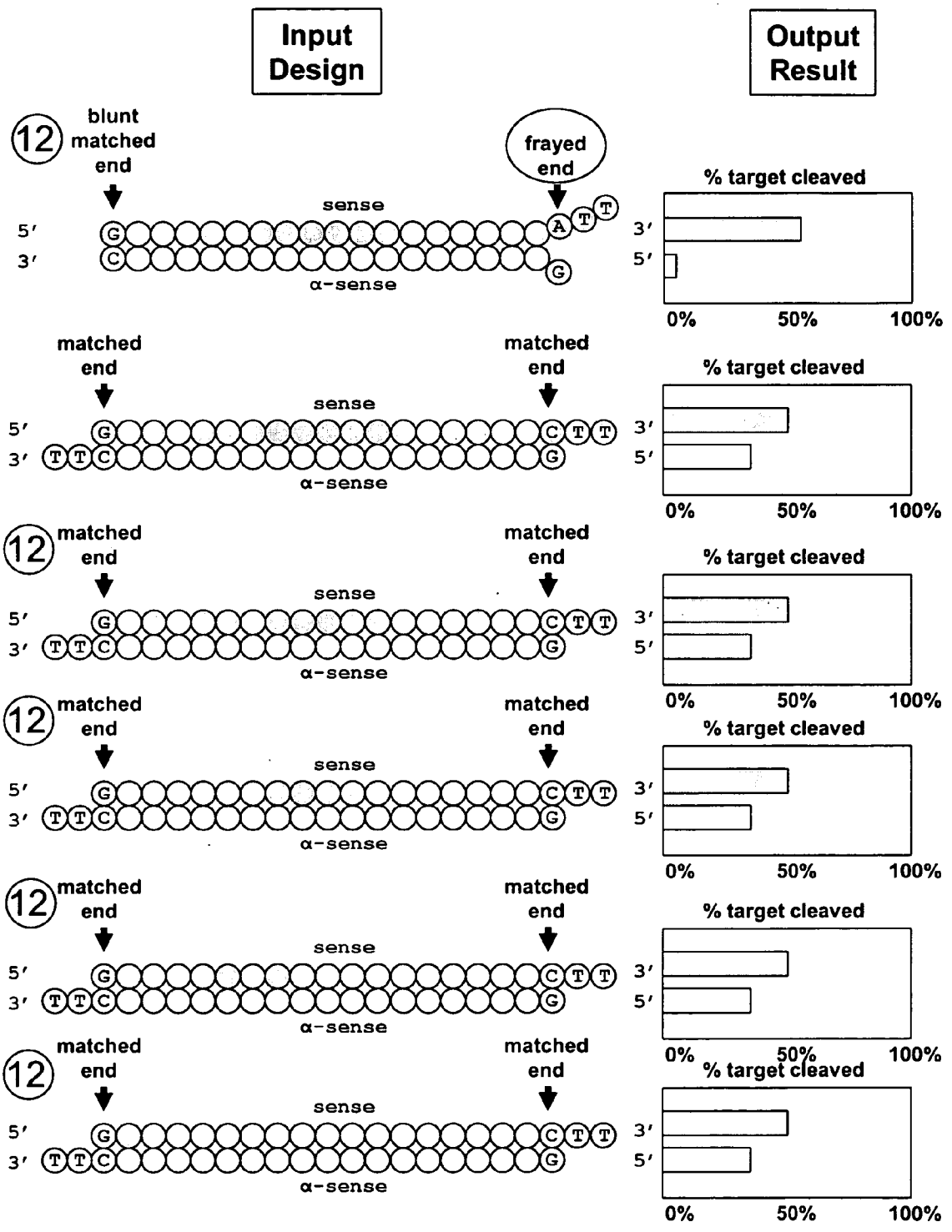

Input Design
Output Result
12
blunt matched end
frayed end
sense
α-sense
5′
3′
% target cleaved
0%
50%
100%
matched end

Fig. 3-1

Blunt-ended siRNAs against WSpET SOD sense

5′ GAGACUUGGGCAAUGUGAC 3′ **(SEQ ID NO.: 7)** (19.31)
.....
3′ TTCUCUGAACCCGUUACACUG 5′ **(SEQ ID NO.: 8)** (SODp11,40)
(wT) 5′ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3′**(SEQ ID NO.: 9)**

5′ GAGACUUGGGCAAUGUGAC 3′ **(SEQ ID NO.: 10)** (19.31)
.....
3′ TTCUCUGAACCCGUUACACUU 5′ **(SEQ ID NO.: 11)** (21.232)
(wT) 5′ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3′**(SEQ ID NO.: 12)**

5′ GAGACUUGGGCAAUGUGAC 3′ **(SEQ ID NO.: 13)** (19.31)
.....
3′ TTCUCUGAACCCGUUACACUI 5′ **(SEQ ID NO.: 14)** (21.246)
(wT) 5′ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3′**(SEQ ID NO.: 15)**

5′ GAGACUUGGGCAAUGUGAC 3′ **(SEQ ID NO.: 16)** (19.31)
.....
3′ TTAUCUGAACCCGUUACACUG 5′ **(SEQ ID NO.: 17)** (21.252)
(wT) 5′ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3′**(SEQ ID NO.: 18)**

5′ GAGACUUGGGCAAUGUGAA 3′ **(SEQ ID NO.: 19)** (19.32)
.....
3′ TTCUCUGAACCCGUUACACUG 5′ **(SEQ ID NO.: 20)** (SODp11,40)
(wT) 5′ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3′**(SEQ ID NO.: 21)**

5′ GAGACUUGGGCAAUGUGAA 3′ **(SEQ ID NO.: 22)** (19.32)
.....
3′ TTCUCUGAACCCGUUACACUU 5′ **(SEQ ID NO.: 23)** (21.232)
(wT) 5′ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3′**(SEQ ID NO.: 24)**

5′ GAGACUUGGGCAAUGUGAA 3′ **(SEQ ID NO.: 25)** (19.32)
.....
3′ TTCUCUGAACCCGUUACACUI 5′ **(SEQ ID NO.: 26)** (21.246)
(wT) 5′ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3′**(SEQ ID NO.: 27)**

Fig. 3-2

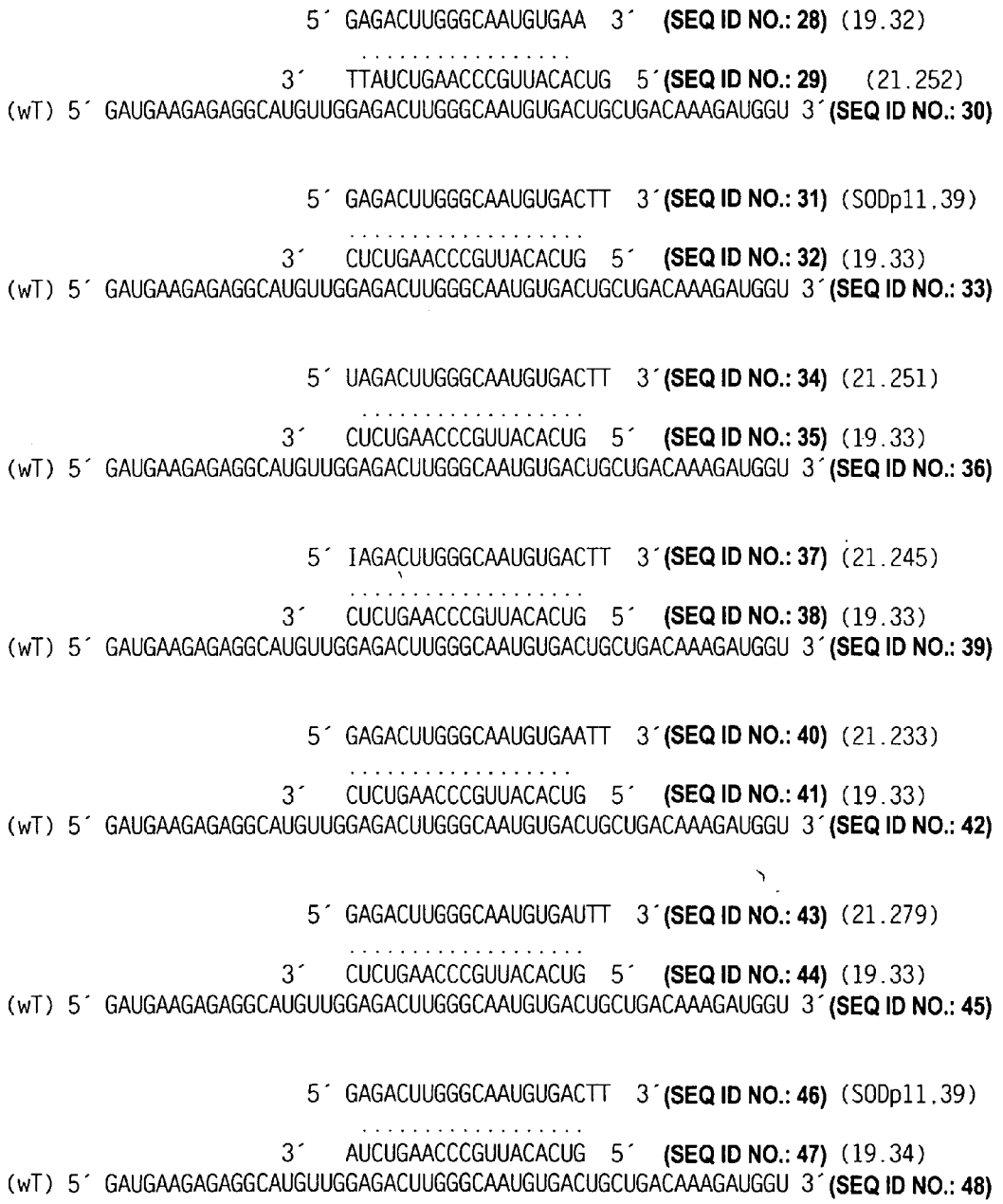

5´ GAGACUUGGGCAAUGUGAA 3´ (SEQ ID NO.: 28) (19.32)
3´ TTAUCUGAACCCGUUACACUG 5´(SEQ ID NO.: 29) (21.252)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´(SEQ ID NO.: 30)
5´ GAGACUUGGGCAAUGUGACTT 3´(SEQ ID NO.: 31) (SODp11.39)
3´ CUCUGAACCCGUUACACUG 5´ (SEQ ID NO.: 32) (19.33)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´(SEQ ID NO.: 33)
5´ UAGACUUGGGCAAUGUGACTT 3´(SEQ ID NO.: 34) (21.251)
3´ CUCUGAACCCGUUACACUG 5´ (SEQ ID NO.: 35) (19.33)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´(SEQ ID NO.: 36)
5´ IAGACUUGGGCAAUGUGACTT 3´(SEQ ID NO.: 37) (21.245)
3´ CUCUGAACCCGUUACACUG 5´ (SEQ ID NO.: 38) (19.33)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´(SEQ ID NO.: 39)
5´ GAGACUUGGGCAAUGUGAATT 3´(SEQ ID NO.: 40) (21.233)
3´ CUCUGAACCCGUUACACUG 5´ (SEQ ID NO.: 41) (19.33)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´(SEQ ID NO.: 42)
5´ GAGACUUGGGCAAUGUGAUTT 3´(SEQ ID NO.: 43) (21.279)
3´ CUCUGAACCCGUUACACUG 5´ (SEQ ID NO.: 44) (19.33)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´(SEQ ID NO.: 45)
5´ GAGACUUGGGCAAUGUGACTT 3´(SEQ ID NO.: 46) (SODp11.39)
3´ AUCUGAACCCGUUACACUG 5´ (SEQ ID NO.: 47) (19.34)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´(SEQ ID NO.: 48)

Fig. 3-3

5´ IAGACUUGGGCAAUGUGACTT 3´**(SEQ ID NO.: 49)** (21.245)
. . . . . .
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.: 50)** (19.34)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´**(SEQ ID NO.: 51)**

5´ GAGACUUGGGCAAUGUGAATT 3´**(SEQ ID NO.: 52)** (21.233)
. . . . . .
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.: 53)** (19.34)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´**(SEQ ID NO.: 54)**

5´ GAGACUUGGGCAAUGUGAUTT 3´**(SEQ ID NO.: 55)** (21.279)
. . . . . .
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.: 56)** (19.34)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´**(SEQ ID NO.: 57)**

Blunt-ended siRNAs (both ends, 19 nts) against WSpET SOD sense

5´ GAGACUUGGGCAAUGUGAC 3´ **(SEQ ID NO.: 58)** (19.31)
. . . . . .
3´ CUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.: 59)** (19.33)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´**(SEQ ID NO.: 60)**

5´ GAGACUUGGGCAAUGUGAC 3´ **(SEQ ID NO.: 61)** (19.31)
. . . . . .
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.: 62)** (19.34)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´**(SEQ ID NO.: 63)**

5´ GAGACUUGGGCAAUGUGAA 3´ **(SEQ ID NO.: 64)** (19.32)
. . . . . .
3´ CUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.: 65)** (19.33)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´**(SEQ ID NO.: 66)**

5´ GAGACUUGGGCAAUGUGAA 3´ **(SEQ ID NO.: 67)** (19.32)
. . . . . .
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.: 68)** (19.34)
(wT) 5´ GAUGAAGAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU 3´**(SEQ ID NO.: 69)**

Fig. 3-4

Blunt-ended siRNAs against WSpET SOD anti-sense

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.: 70)**
5´ GAGACUUGGGCAAUGUGAC 3´ **(SEQ ID NO.: 71)** (19.31)
.....
3´ TTCUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.: 72)** (SODp11,40)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.: 73)**
5´ GAGACUUGGGCAAUGUGAC 3´ **(SEQ ID NO.: 74)** (19.31)
.....
3´ TTCUCUGAACCCGUUACACUU 5´ **(SEQ ID NO.: 75)** (21.232)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.: 76)**
5´ GAGACUUGGGCAAUGUGAC 3´ **(SEQ ID NO.: 77)** (19.31)
.....
3´ TTCUCUGAACCCGUUACACUI 5´ **(SEQ ID NO.: 78)** (21.246)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.: 79)**
5´ GAGACUUGGGCAAUGUGAC 3´ **(SEQ ID NO.: 80)** (19.31)
.....
3´ TTAUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.: 81)** (21.252)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.: 82)**
5´ GAGACUUGGGCAAUGUGAA 3´ **(SEQ ID NO.: 83)** (19.32)
.....
3´ TTCUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.: 84)** (SODp11,40)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.: 85)**
5´ GAGACUUGGGCAAUGUGAA 3´ **(SEQ ID NO.: 86)** (19.32)
.....
3´ TTCUCUGAACCCGUUACACUU 5´ **(SEQ ID NO.: 87)** (21.232)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.: 88)**
5´ GAGACUUGGGCAAUGUGAA 3´ **(SEQ ID NO.: 89)** (19.32)
.....
3´ TTCUCUGAACCCGUUACACUI 5´ **(SEQ ID NO.: 90)** (21.246

Fig. 3-5

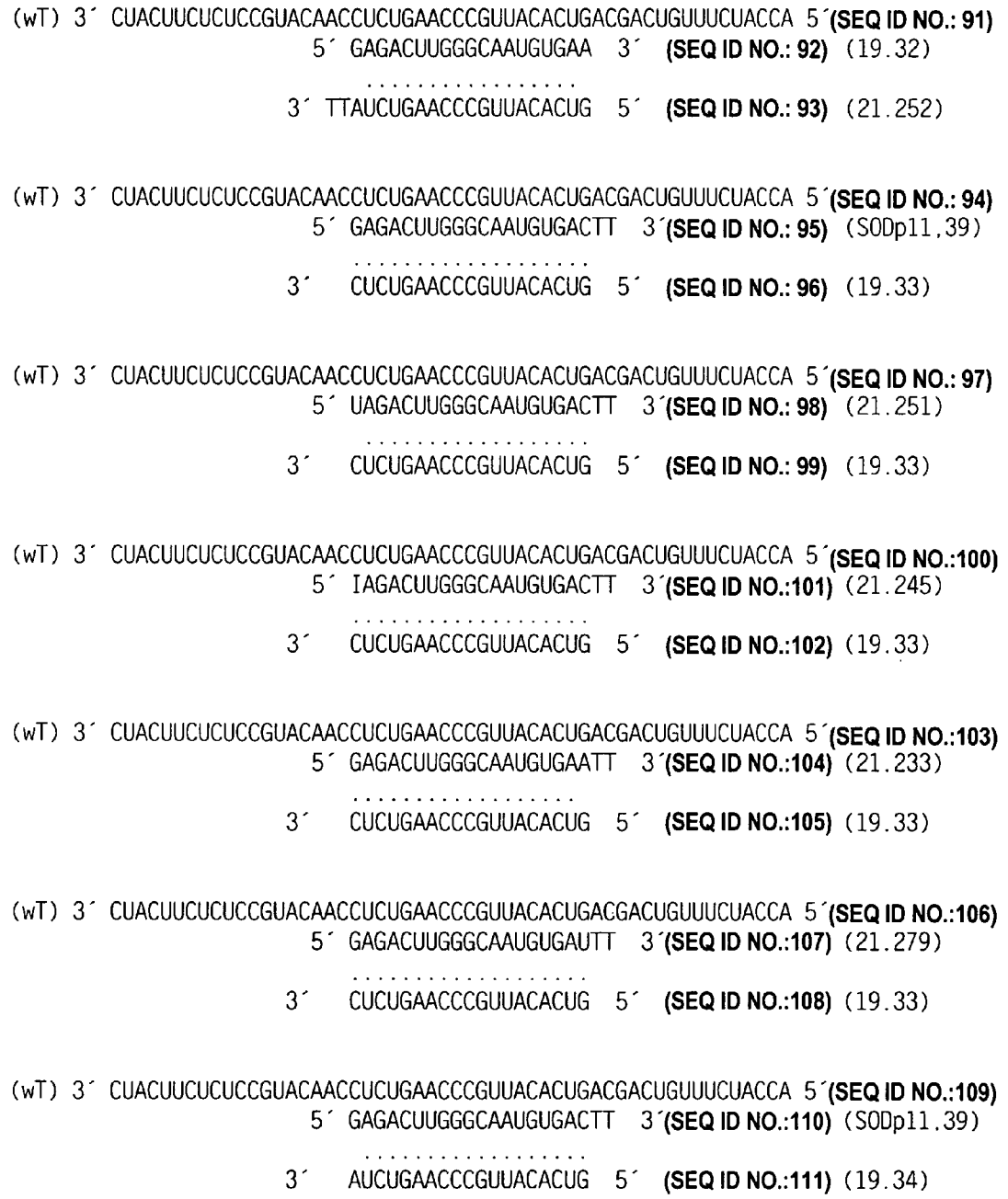

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´(SEQ ID NO.: 91)
5´ GAGACUUGGGCAAUGUGAA 3´ (SEQ ID NO.: 92) (19.32)
3´ TTAUCUGAACCCGUUACACUG 5´ (SEQ ID NO.: 93) (21.252)
(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´(SEQ ID NO.: 94)
5´ GAGACUUGGGCAAUGUGACTT 3´(SEQ ID NO.: 95) (SODp11,39)
3´ CUCUGAACCCGUUACACUG 5´ (SEQ ID NO.: 96) (19.33)
(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´(SEQ ID NO.: 97)
5´ UAGACUUGGGCAAUGUGACTT 3´(SEQ ID NO.: 98) (21.251)
3´ CUCUGAACCCGUUACACUG 5´ (SEQ ID NO.: 99) (19.33)
(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´(SEQ ID NO.:100)
5´ IAGACUUGGGCAAUGUGACTT 3´(SEQ ID NO.:101) (21.245)
3´ CUCUGAACCCGUUACACUG 5´ (SEQ ID NO.:102) (19.33)
(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´(SEQ ID NO.:103)
5´ GAGACUUGGGCAAUGUGAATT 3´(SEQ ID NO.:104) (21.233)
3´ CUCUGAACCCGUUACACUG 5´ (SEQ ID NO.:105) (19.33)
(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´(SEQ ID NO.:106)
5´ GAGACUUGGGCAAUGUGAUTT 3´(SEQ ID NO.:107) (21.279)
3´ CUCUGAACCCGUUACACUG 5´ (SEQ ID NO.:108) (19.33)
(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´(SEQ ID NO.:109)
5´ GAGACUUGGGCAAUGUGACTT 3´(SEQ ID NO.:110) (SODp11.39)
3´ AUCUGAACCCGUUACACUG 5´ (SEQ ID NO.:111) (19.34)

Fig. 3-6

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.:112)**
5´ UAGACUUGGGCAAUGUGACTT 3´**(SEQ ID NO.:113)** (21.251)
.....
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.:114)** (19.34)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.:115)**
5´ IAGACUUGGGCAAUGUGACTT 3´**(SEQ ID NO.:116)** (21.245)
.....
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.:117)** (19.34)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.:118)**
5´ GAGACUUGGGCAAUGUGAATT 3´**(SEQ ID NO.:119)** (21.233)
.....
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.:120)** (19.34)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.:121)**
5´ GAGACUUGGGCAAUGUGAUTT 3´**(SEQ ID NO.:122)** (21.279)
.....
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.:123)** (19.34)

Fig. 3-7

Blunt-ended siRNAs (both ends, 19 nts) against WSpET SOD anti-sense

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.:124)**
5´ GAGACUUGGGCAAUGUGAC 3´ **(SEQ ID NO.:125)** (19.31)
.....
3´ CUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.:126)** (19.33)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.:127)**
5´ GAGACUUGGGCAAUGUGAC 3´ **(SEQ ID NO.:128)** (19.31)
.....
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.:129)** (19.34)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.:130)**
5´ GAGACUUGGGCAAUGUGAA 3´ **(SEQ ID NO.:131)** (19.32)
.....
3´ CUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.:132)** (19.33)

(wT) 3´ CUACUUCUCUCCGUACAACCUCUGAACCCGUUACACUGACGACUGUUUCUACCA 5´**(SEQ ID NO.:133)**
5´ GAGACUUGGGCAAUGUGAA 3´ **(SEQ ID NO.:134)** (19.32)
.....
3´ AUCUGAACCCGUUACACUG 5´ **(SEQ ID NO.:135)** (19.34)

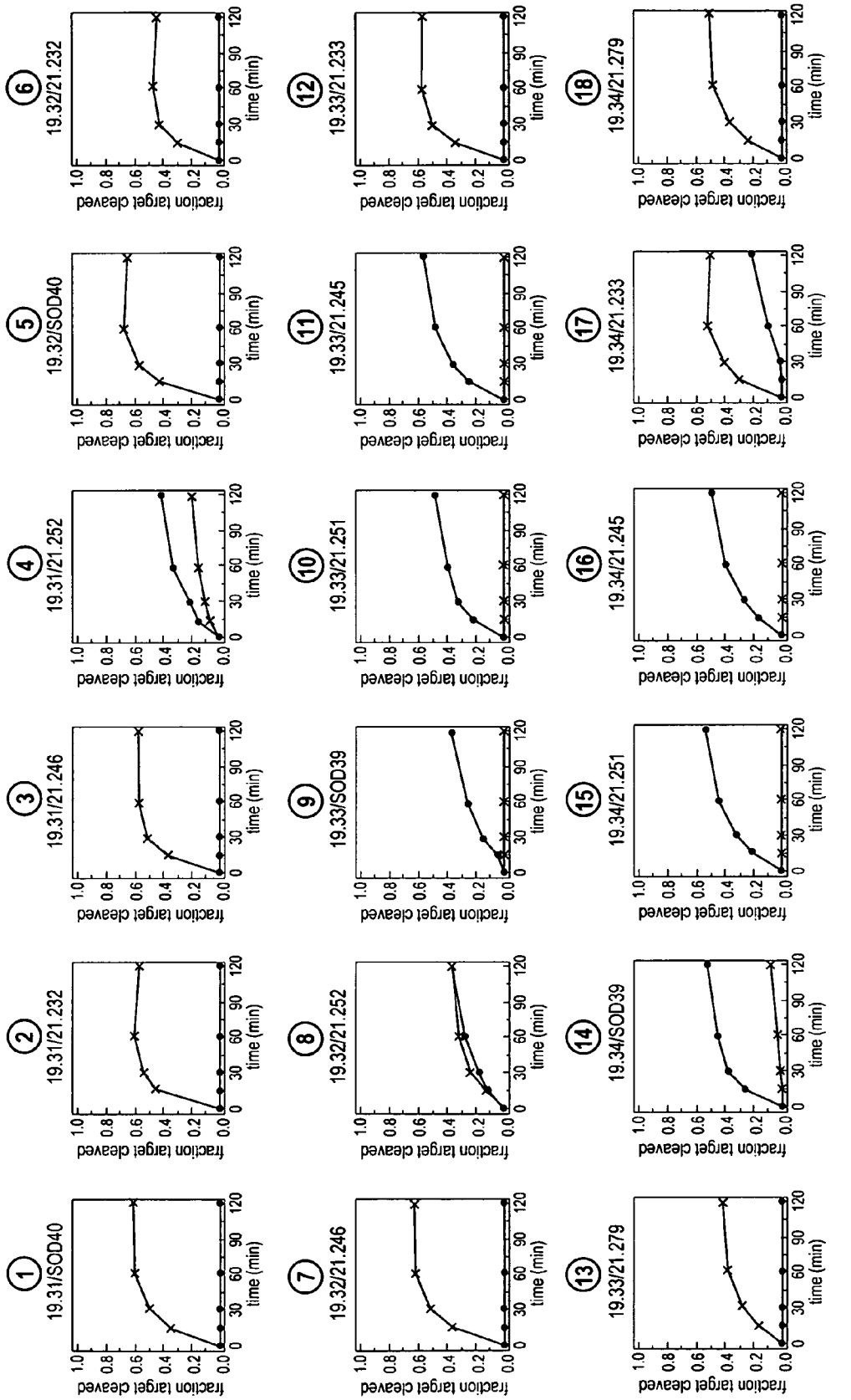
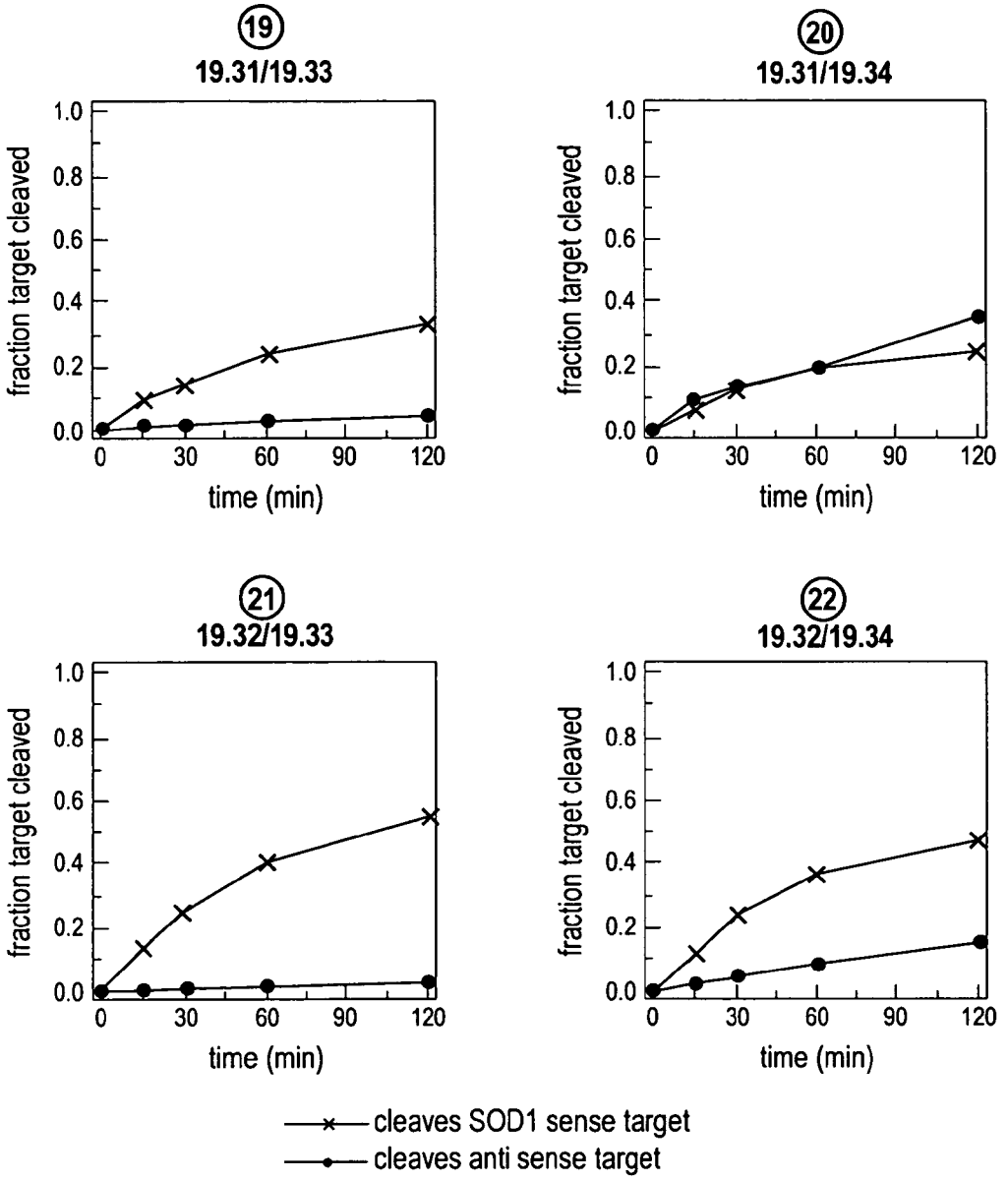

1 19.31/SOD40
2 19.31/21.232
3 19.31/21.246
4 19.31/21.252
5 19.32/SOD40
6 19.32/21.232
7 19.32/21.246
8 19.32/21.252
9 19.33/SOD39
10 19.33/21.251
11 19.33/21.245
12 19.33/21.233
13 19.33/21.279
14 19.34/SOD39
15 19.34/21.251
16 19.34/21.245
17 19.34/21.233
18 19.34/21.279
fraction target cleaved
time (min)
0.0
0.2
0.4
0.6
0.8
1.0
0
30
60
90
120
cleaves SOD1 sense target
cleaves anti sense target

Fig. 4

Fig. 5


19
19.31/19.33
20
19.31/19.34
21
19.32/19.33
22
19.32/19.34
fraction target cleaved
time (min)
0.0
0.2
0.4
0.6
0.8
1.0
0
30
60
90
120
cleaves SOD1 sense target
cleaves anti sense target

METHODS AND COMPOSITIONS FOR ENHANCING THE EFFICACY AND SPECIFICITY OF SINGLE AND DOUBLE BLUNT-ENDED SIRNA

RELATED APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Application Ser. No. 60/532,116, entitled "Methods and Compositions for Enhancing the Efficacy and Specificity of Single and Double Blunt-Ended siRNA", filed Dec. 22, 2003. The entire contents of the above-referenced provisional patent application are incorporated herein by this reference.

RELATED INFORMATION

[0002] The contents of any patents, patent applications, and references cited throughout this specification are hereby incorporated by reference in their entireties.

BACKGROUND OF THE INVENTION

[0003] Small interfering RNAs (siRNAs) are produced by the cleavage of double-stranded RNA (dsRNA) precursors by Dicer, a member of the RNase III family of dsRNA-specific endonucleases. Typically, siRNAs result when transposons, viruses, or endogenous genes express long dsRNA or when dsRNA is introduced experimentally into plant or animal cells to trigger gene silencing, a process known as RNA interference (RNAi).

[0004] siRNAs were first identified as the specificity determinants of the RNA interference (RNAi) pathway, where they act as guides to direct endonucleolytic cleavage of their target RNAs. Prototypical siRNA duplexes are 21 nucleotide, double-stranded RNAs that contain 19 base pairs, with two-nucleotide, 3' overhanging ends. Active siRNAs contain 5' phosphates and 3' hydroxyls.

[0005] siRNAs are typically found in the RNA-induced silencing complex (RISC) that mediates both cleavage and translational control. siRNA duplexes can assemble into RISC in the absence of target mRNA, both in vivo and in vitro. Each RISC contains only one of the two strands of the siRNA duplex. Since siRNA duplexes have no foreknowledge of which siRNA strand will guide target cleavage, both strands must assemble with the appropriate proteins to form a RISC.

[0006] It has been observed that both siRNA strands are competent to direct RNAi (Tuschl et al., *Genes Dev* 13, 3191-3197 (1999); Hammond et al., *Nature* 404, 293-296 (2000); Zamore et al., *Cell* 101, 25-33 (2000); Elbashir et al., *Genes Dev* 15, 188-200 (2001); Elbashir et al., *EMBO J* 20, 6877-6888 (2001); Nykänen et al., *Cell* 107, 309-321 (2001). That is, the antisense strand of an siRNA can direct cleavage of a corresponding sense RNA target, whereas the sense siRNA strand directs cleavage of an antisense target. In this way, siRNA duplexes appear to be functionally symmetric.

[0007] The ability to control which strand of an siRNA duplex enters into the RISC complex to direct cleavage of a corresponding RNA target would provide a significant advance for both research and therapeutic applications of RNAi technology.

SUMMARY OF THE INVENTION

[0008] The invention solves the foregoing problems of siRNA gene targeting by determining the structural and functional characteristics of single and blunt-ended siRNAs and in particular, their strand specificity for a gene target. Accordingly, an entirely new constellation of single and double blunt-ended siRNA agents, e.g., siRNA duplexes, can be designed to efficiently and specifically modulate a sense and/or antisense gene target.

[0009] In addition, the invention provides a method for introducing alterations in either the 5', 3', or both the 5' and 3' of a single or double blunt-ended siRNA such that either the sense, the antisense, or both the sense and antisense strand will enter the RNAi pathway (e.g., RISC) and target a cognate gene target(s) for cleavage and destruction. Typically, the alteration takes the form of a mismatched base pair that allows for a portion of the siRNA duplex, e.g., the 5' end of the antisense strand, to separate or fray.

[0010] Accordingly, the invention has several advantages which include, but are not limited to, the following:

[0011] providing methods for designing single and double blunt-ended siRNA agents, e.g., siRNA duplexes, have a characteristic strand specificity;

[0012] providing single and double blunt-ended siRNA agents, e.g., siRNA duplexes or small hairpin RNAs (shRNAs) with at least one blunt end, suitable for gene modulation in plant or animal cells; and

[0013] methods for modulating gene expression in a subject in need thereof using the single or double blunt-ended siRNA compositions of the invention, e.g., in the form of a pharmaceutical composition suitable for administering to a patient.

[0014] Accordingly, in one aspect, the invention provides methods for improving the efficiency (or specificity) of an RNAi reaction comprising modifying (e.g., increasing) the asymmetry of an RNAi agent (i.e., an RNA duplex having at least one blunt end) such that the ability of the sense or second strand to mediate RNAi (e.g., mediate cleavage of a target RNA) is lessened.

[0015] In one embodiment, the asymmetry is increased in favor of the 5' end of the first strand, e.g., by lessening the bond strength (e.g., the strength of the interaction) between the 5' end of the first strand and 3' end of the second strand relative to the bond strength (e.g., the strength of the interaction) between the 5' end of the second strand and the 3' end of the first strand.

[0016] In another embodiment, the asymmetry is increased in favor of the 5' end of the first strand by increasing bond strength (e.g., the strength of the interaction) between the 5' end of the second or sense strand and the 3' end of the first or antisense strand, relative to the bond strength (e.g., the strength of the interaction) between the 5' end of the first and the 3' end of the second strand.

[0017] In another embodiment, the bond strength is increased, e.g., the hydrogen bonding is increased between nucleotides or analogs at the 5' end, e.g., within 5 nucleotides of the second or sense strand (numbered from the 5' end of the second strand) and complementary nucleotides of the first or antisense strand. It is understood that the asymmetry can be zero (i.e., no asymmetry), for example, when the bonds or base pairs between the 5' and 3' terminal bases are of the same nature, strength or structure. More routinely, however, there exists some asymmetry due to the different

nature, strength or structure of at least one nucleotide (often one or more nucleotides) between terminal nucleotides or nucleotide analogs.

[0018] Accordingly, in one aspect, the instant invention provides a method of enhancing the ability of a first strand of a single or double blunt-ended RNAi agent to act as a guide strand in mediating RNAi, involving lessening the base pair strength between the 5' end of the first strand and the 3' end of a second strand of the duplex as compared to the base pair strength between the 3' end of the first strand and the 5' end of the second strand.

[0019] In a related aspect, the invention provides a method of enhancing the efficacy of a single or double blunt-ended siRNA duplex, the siRNA duplex comprising a sense and an antisense strand, involving lessening the base pair strength between the antisense strand 5' end (AS 5') and the sense strand 3' end (S 3') as compared to the base pair strength between the antisense strand 3' end (AS 3') and the sense strand 5' end (S '5), such that efficacy is enhanced.

[0020] In another aspect of the invention, a method is provided for promoting entry of a desired strand of an single or double blunt-ended siRNA duplex into a RISC complex, comprising enhancing the asymmetry of the single or double blunt-ended siRNA duplex, such that entry of the desired strand is promoted. In one embodiment of this aspect of the invention, the asymmetry is enhanced by lessening the base pair strength between the 5' end of the desired strand and the 3' end of a complementary strand of the duplex as compared to the base pair strength between the 3' end of the desired strand and the 5' end of the complementary strand.

[0021] In another aspect of the invention, a single or double blunt-ended siRNA duplex is provided comprising a sense strand and an antisense strand, wherein the base pair strength between the antisense strand 5' end (AS 5') and the sense strand 3' end (S 3') is less than the base pair strength between the antisense strand 3' end (AS 3') and the sense strand 5' end (S '5), such that the antisense strand preferentially guides cleavage of a target mRNA.

[0022] In one embodiment of these aspects of the invention, the base-pair strength is less due to fewer G:C base pairs between the 5' end of the first or antisense strand and the 3' end of the second or sense strand than between the 3' end of the first or antisense strand and the 5' end of the second or sense strand.

[0023] In another embodiment, the base pair strength is less due to at least one mismatched base pair between the 5' end of the first or antisense strand and the 3' end of the second or sense strand. Preferably, the mismatched or wobble base pair is selected from the group consisting of G:A, C:A, C:U, G:G, A:A, C:C, U:U, I:A, I:U, and I:C.

[0024] In yet another embodiment, the base pair strength is less due to at least one base pair comprising a modified nucleotide. In preferred embodiments, the modified nucleotide is selected from the group consisting of 2-amino-G (e.g., 2,2-diamino-1,2-dihydro-purin-6-one), 2-amino-A, 2,6-diamino-G, and 2,6-diamino-A.

[0025] In other embodiments of the above aspects, the single or double blunt-ended RNAi agent or siRNA duplex is derived from an engineered precursor, and can be chemically synthesized or enzymatically synthesized.

[0026] In another aspect of the instant invention, compositions are provided comprising a single or double blunt-ended siRNA duplex of the invention formulated to facilitate entry of the siRNA duplex into a cell. Also provided are pharmaceutical composition comprising a siRNA duplex of the invention.

[0027] Further provided are an engineered pre-miRNA comprising the siRNA duplex of any one of the preceding claims, as well as a vector encoding the pre-miRNA. In related aspects, the invention provides a pre-miRNA comprising the pre-miRNA, as well as a vector encoding the pre-miRNA.

[0028] Also featured in the instant invention are small hairpin RNA (shRNA) capable of forming at least a single blunt end comprising nucleotide sequence identical to the sense and antisense strand of the siRNA duplex as described above.

[0029] In one embodiment, the nucleotide sequence identical to the sense strand is upstream of the nucleotide sequence identical to the antisense strand. In another embodiment, the nucleotide sequence identical to the antisense strand is upstream of the nucleotide sequence identical to the sense strand. Further provided are vectors and transgenes encoding the shRNAs of the invention.

[0030] In yet another aspect, the invention provides cells comprising the vectors featured in the instant invention. Preferably, the cell is a mammalian cell, e.g., a human cell.

[0031] In other aspects of the invention, methods of enhancing silencing of a target mRNA, comprising contacting a cell having an RNAi pathway with any of the foregoing single or double blunt-ended RNAi agents such that silencing is enhanced.

[0032] Also provided are methods of enhancing silencing of a target mRNA in a subject, comprising administering to the subject a pharmaceutical composition comprising any of the foregoing single or double blunt-ended RNAi agents such that silencing is enhanced.

[0033] Further provided is a method of decreasing silencing of an inadvertent target mRNA by a single or double blunt-ended RNAi agents the RNAi agent comprising a sense strand and an antisense strand involving the steps of: (a) detecting a significant degree of complementarity between the sense strand and the inadvertent target; and (b) enhancing the base pair strength between the 5' end of the sense strand and the 3' end of the antisense strand relative to the base pair strength between the 3' end of the sense strand and the 5' end of the antisense strand; such that silencing of the inadvertent target mRNA is decreased. In a preferred embodiment, the silencing of the inadvertent target mRNA is decreased relative to silencing of a desired target mRNA.

[0034] Other features and advantages of the invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] **FIG. 1** shows a schematic of the structural and functional characteristics of classical siRNA (i.e., having 3' dinucleotide overhangs) with either a 5' or 3' frayed end as compared to the siRNAs of the invention having at least one blunt end. Selected single blunt-ended siRNAs with either a

5' or 3' frayed end are shown as well as their corresponding ability to target cleavage of a test sense and/or antisense target. Numbers on the left correspond to the siRNA shown in further detail structurally in **FIG. 4** and as tested for target specificity in **FIG. 5**.

[0036] **FIG. 2** shows a schematic of the structural and functional characteristics of siRNAs of the invention having both 5' and 3' blunt ends. Selected double blunt-ended siRNAs with either a 5' or 3' frayed end are shown as well as their corresponding ability to target cleavage of a sense and/or antisense gene target. Numbers on the left correspond to the siRNA shown in further detail structurally in **FIG. 4** and tested for target specificity in **FIG. 5**.

[0037] **FIG. 3** shows the structure of all siRNA duplexes tested, in particular, the single and double blunt-ended siRNA duplexes of the invention and their correspondence with sense or antisense gene targets to determine their efficacy and specificity. Each siRNA duplex tested is identified by a number which corresponds to functional target specificity results obtained in vitro using *Drosophila* extracts (and shown in **FIG. 5**). Single blunt-ended siRNA duplexes and double blunted-ended siRNA duplexes and their alignment with sense targets are numbered, respectively, 1-18 and 19-22. The foregoing and their alignment with antisense targets are numbered, respectively, 23-44.

[0038] **FIG. 4** shows the efficacy and specificity of the single blunt-ended siRNA duplexes and their ability to cleave sense and antisense gene targets using *Drosophila* extracts that provide a functional RISC-mediated RNAi pathway. Black (x) data points show % antisense gene target cleaved (SOD1 sense target; i.e., gene knockdown) whereas red (o) data points show % sense gene target cleaved.

[0039] **FIG. 5** shows the efficacy and specificity of the double blunt-ended siRNA duplexes and their ability to cleave sense and antisense gene targets using *Drosophila* extracts that provide a functional RISC-mediated RNAi pathway. Black (x) data points show % antisense gene target cleaved (SOD1 sense target; i.e., gene knockdown) whereas red (o) data points show % sense gene target cleaved.

DETAILED DESCRIPTION OF THE INVENTION

[0040] In order to provide a clear understanding of the specification and claims, the following definitions are conveniently provided below.

Definitions

[0041] As used herein the term "blunt end", for example, "single blunt-end" or "double blunt-ended siRNA" refers to, e.g., an siRNA duplex where at least one end of the duplex lacks any overhang, e.g., a 3' dinucleotide overhang, such that both the 5' and 3' strand end together, i.e., are flush or as referred to herein, are blunt. The molecules of the invention have at least one blunt end and, preferably, two blunt ends, i.e., are double blunt-ended (See **FIGS. 1-3** which show schematically classical siRNA duplexes having 3' dinucleotide overhangs as compared with the single and double blunt-ended siRNAs of the invention).

[0042] The term "small interfering RNA" ("siRNA") (also referred to in the art as "short interfering RNAs") refers to an RNA (or RNA analog) comprising between about 10-50 nucleotides (or nucleotide analogs) which is capable of directing or mediating RNA interference. Preferably, an siRNA comprises between about 15-30 nucleotides or nucleotide analogs, more preferably between about 16-25 nucleotides (or nucleotide analogs), even more preferably between about 18-23 nucleotides (or nucleotide analogs), and even more preferably between about 19-22 nucleotides (or nucleotide analogs) (e.g., 19, 20, 21 or 22 nucleotides or nucleotide analogs). As mentioned above, at least one end if not both ends of the siRNA of the invention, is blunt. Preferred single blunt-ended siRNA molecules comprise a 21 nucleotide (nt) strand paired with a strand that is 19 nt, 18 nt, or 17 nt. In another embodiment, single blunt-ended siRNA molecules comprise a 19 nt strand paired with a 18 nt strand or, preferably, a 17 nt strand, wherein the 19 nt strand is favored to enter the RISC pathway. It is also understood that a blunt ended siRNA, if base paired or matched, is more prone to separating or fraying, then an end that is matched but also has a one or more nucleotide overhang, e.g., a dinucleotide overhang, because of the unpaired helical nature of the overhang and the stacking forces which contribute to maintaining the base pairs immediately downstream.

[0043] The term "RNA interference" ("RNAi") (also referred to in the art as "gene silencing" and/or "target silencing", e.g., "target mRNA silencing") refers to a selective intracellular degradation of RNA. RNAi occurs in cells naturally to remove foreign RNAs (e.g., viral RNAs). Natural RNAi proceeds via fragments cleaved from free dsRNA which direct the degradative mechanism to other similar RNA sequences. Alternatively, RNAi can be initiated by the hand of man, for example, to silence the expression of target genes.

[0044] The term "antisense strand" of an siRNA or RNAi agent refers to a strand that is substantially complementary to a section of about 10-50 nucleotides, e.g., about 15-30, 16-25, 18-23 or 19-22 nucleotides of the mRNA of the gene targeted for silencing. The antisense strand or first strand has sequence sufficiently complementary to the desired target mRNA sequence to direct target-specific RNA interference (RNAi), e.g., complementarity sufficient to trigger the destruction of the desired target mRNA by the RNAi machinery or process.

[0045] The term "sense strand" or "second strand" of an siRNA or RNAi agent refers to a strand that is complementary to the antisense strand or first strand. Antisense and sense strands can also be referred to as first or second strands, the first or second strand having complementarity to the target sequence and the respective second or first strand having complementarity to said first or second strand.

[0046] The term "guide strand" refers to a strand of an RNAi agent, e.g., an antisense strand of an siRNA duplex, that enters into the RISC complex and directs cleavage of the target mRNA.

[0047] The term "target gene" is a gene whose expression is to be selectively inhibited or "silenced". This silencing is achieved by cleaving the mRNA of the target gene by an siRNA or miRNA, e.g., an siRNA or miRNA that is created from an engineered RNA precursor by a cell's RNAi system. One portion or segment of a duplex stem of the RNA precursor is an antisense strand that is complementary, e.g., sufficiently complementary to trigger the destruction of the

desired target mRNA by the RNAi machinery or process, to a section of about 18 to about 40 or more nucleotides of the mRNA of the target gene.

[0048] The term "asymmetry", as in the asymmetry of a single or double blunt-ended siRNA duplex, refers to an inequality of bond strength or base pairing strength between the siRNA termini (e.g., between terminal nucleotides on a first strand and terminal nucleotides on an opposing second strand, e.g., a base pair mismatch that allows for a separation or fraying of the end(s)), such that the 5' end of one strand of the duplex is more frequently in a transient unpaired, e.g., single-stranded, state than the 5' end of the complementary strand. This structural difference determines that one strand of the duplex is preferentially incorporated into a RISC complex. The strand whose 5' end is less tightly paired to the complementary strand will preferentially be incorporated into RISC and mediate RNAi.

[0049] The term "bond strength" or "base pair strength" refers to the strength of the interaction between pairs of nucleotides (or nucleotide analogs) on opposing strands of an oligonucleotide duplex (e.g., an siRNA duplex), due primarily to hydrogen-bonding, Van der Waals interactions, and the like between such nucleotides (or nucleotide analogs).

[0050] The term "fray" or "fraying" refers to the ability of a portion of the siRNA duplex of the invention to separate, typically at the end, preferably at the 5' end of the first or antisense strand, due to a base pair mismatch. For determining the thermodynamic stability or local thermodynamic stability of such ends, energy rules can be based on nearest neighbor analysis and/or amount of stacking.

DETAILED DESCRIPTION

Overview

[0051] The present invention features "small interfering RNA molecules" ("siRNA molecules" or "siRNA") having at least one blunt end, methods of making such siRNA molecules and methods for using the single or double blunt-ended siRNA molecules (e.g., research and/or therapeutic methods). A blunt-ended siRNA molecule of the invention is a duplex consisting of a sense strand and complementary antisense strand, the antisense strand having sufficient complementarity to a target mRNA to mediate RNAi and having at least one end (5', 3', or both 5' and 3') without an overhang. Accordingly, the molecules of the invention are distinguished from typical siRNA molecules which have a 3' dinucleotide overhang at each end of the molecule.

[0052] Preferably, the strands are aligned such that, at one end, preferably at both ends, there are no bases at the end of the strands which do not align (i.e., for which no complementary bases occur in the opposing strand) such that no overhang occurs at one or both ends of the duplex when the strands are annealed. Preferably, the single or double blunt-ended siRNA molecule has a length from about 10-50 or more nucleotides, i.e., each strand comprises 10-50 nucleotides (or nucleotide analogs). More preferably, the siRNA molecule has a length from about 15-45 or 15-30 nucleotides. Even more preferably, the siRNA molecule has a length from about 16-25 nucleotides, 18-23 nucleotides, or 19 nucleotides. The single or double blunt-ended siRNA molecules of the invention further have a sequence that is "sufficiently complementary" to a target mRNA sequence to direct target-specific RNA interference (RNAi), as defined herein, i.e., the single or double blunt-ended siRNA has a sequence sufficient to trigger the destruction of the target mRNA by the RNAi machinery or process.

1. Preferred RNA Molecules

[0053] The single or double blunt-ended siRNAs featured in the invention provide enhanced specificity and efficacy for mediating RISC-mediated cleavage of a desired target gene. In a preferred aspect, the base pair strength between the antisense strand 5' end (AS 5') and the sense strand 3' end (S 3') of the siRNAs is less than the bond strength or base pair strength between the antisense strand 3' end (AS 3') and the sense strand 5' end (S '5), such that the antisense strand preferentially guides cleavage of a target mRNA. In one embodiment, the bond strength or base-pair strength is less due to fewer G:C base pairs between the 5' end of the first or antisense strand and the 3' end of the second or sense strand than between the 3' end of the first or antisense strand and the 5' end of the second or sense strand.

[0054] In another embodiment, the bond strength or base pair strength is less due to at least one mismatched base pair between the 5' end of the first or antisense strand and the 3' end of the second or sense strand. Preferably, the mismatched base pair is selected from the group consisting of G:A, C:A, C:U, G:G, A:A, C:C and U:U. In a related embodiment, the bond strength or base pair strength is less due to at least one wobble base pair, e.g., G:U, between the 5' end of the first or antisense strand and the 3' end of the second or sense strand.

[0055] In yet another embodiment, the bond strength or base pair strength is less due to at least one base pair comprising a rare nucleotide, e.g., inosine (I). Preferably, the base pair is selected from the group consisting of an I:A, I:U, and I:C.

[0056] In yet another embodiment, the bond strength or base pair strength is less due to at least one base pair comprising a modified nucleotide. In preferred embodiments, the modified nucleotide is selected from the group consisting of 2-amino-G, 2-amino-A, 2,6-diamino-G, and 2,6-diamino-A.

[0057] In general, single or double blunt-ended siRNAs containing nucleotide sequences sufficiently identical to a portion of the target gene to effect RISC-mediated cleavage of the target gene are preferred. 2. Gene Target Sequence Identity

[0058] Typically, 100% sequence identity between the single or double blunt-ended siRNA and the target gene is not required to practice the present invention. The invention has the advantage of being able to tolerate preferred sequence variations of the methods and compositions of the invention in order to enhance efficiency and specificity of RNAi. For example, single or double blunt-ended siRNA sequences with insertions, deletions, and single point mutations relative to the target sequence can also be effective for inhibition. Alternatively, single or double blunt-ended siRNA sequences with nucleotide analog substitutions or insertions can be effective for inhibition.

[0059] Sequence identity may be determined by sequence comparison and alignment algorithms known in the art. To

determine the percent identity of two nucleic acid sequences (or of two amino acid sequences), the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the first sequence or second sequence for optimal alignment). The nucleotides (or amino acid residues) at corresponding nucleotide (or amino acid) positions are then compared. When a position in the first sequence is occupied by the same residue as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology=# of identical positions/total # of positions×100), optionally penalizing the score for the number of gaps introduced and/or length of gaps introduced.

[0060] The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In one embodiment, the alignment generated over a certain portion of the sequence aligned having sufficient identity but not over portions having low degree of identity (i.e., a local alignment). A preferred, non-limiting example of a local alignment algorithm utilized for the comparison of sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-68, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-77. Such an algorithm is incorporated into the BLAST programs (version 2.0) of Altschul, et al. (1990) *J. Mol. Biol.* 215:403-10.

[0061] In another embodiment, the alignment is optimized by introducing appropriate gaps and percent identity is determined over the length of the aligned sequences (i.e., a gapped alignment). To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) *Nucleic Acids Res.* 25(17):3389-3402. In another embodiment, the alignment is optimized by introducing appropriate gaps and percent identity is determined over the entire length of the sequences aligned (i.e., a global alignment). A preferred, non-limiting example of a mathematical algorithm utilized for the global comparison of sequences is the algorithm of Myers and Miller, *CABIOS* (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

[0062] Greater than 80% sequence identity, e.g., 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100% sequence identity, between the siRNA antisense strand and the portion of the target gene is preferred. Alternatively, the siRNA may be defined functionally as a nucleotide sequence (or oligonucleotide sequence) that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50° C. or 70° C. hybridization for 12-16 hours; followed by washing). Additional preferred hybridization conditions include hybridization at 70° C. in 1×SSC or 50° C. in 1×SSC, 50% formamide followed by washing at 70° C. in 0.3×SSC or hybridization at 70° C. in 4×SSC or 50° C. in 4×SSC, 50% formamide followed by washing at 67° C. in 1×SSC. The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10° C. less than the melting temperature (Tm) of the hybrid, where Tm is determined according to the following equations. For hybrids less than 18 base pairs in length, Tm(° C.)=2(# of A+T bases)+4(# of G+C bases). For hybrids between 18 and 49 base pairs in length, Tm(° C.)=81.5+16.6(log 10[Na+])+0.41 (% G+C)−(600/N), where N is the number of bases in the hybrid, and [Na+] is the concentration of sodium ions in the hybridization buffer ([Na+] for 1×SSC=0.165 M). Additional examples of stringency conditions for polynucleotide hybridization are provided in Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., chapters 9 and 11, and *Current Protocols in Molecular Biology*, 1995, F. M. Ausubel et al., eds., John Wiley & Sons, Inc., sections 2.10 and 6.3-6.4, incorporated herein by reference. The length of the identical nucleotide sequences may be at least about 10, 12, 15, 17, 20, 22, 25, 27, 30, 32, 35, 37, 40, 42, 45, 47 or 50 bases.

3. Other Modifications for RNA Stability

[0063] The RNA molecules of the present invention can be modified to improve stability in serum or in growth medium for cell cultures. In order to enhance the stability, the 3'-residues may be stabilized against degradation, e.g., they may be selected such that they consist of purine nucleotides, particularly adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNA interference.

[0064] In a preferred aspect, the invention features small interfering RNAs (siRNAs) that include a sense strand and an antisense strand, wherein the antisense strand has a sequence sufficiently complementary to a target mRNA sequence to direct target-specific RNA interference (RNAi) and wherein the sense strand and/or antisense strand is modified by the substitution of internal nucleotides with modified nucleotides, such that in vivo stability is enhanced as compared to a corresponding unmodified siRNA. As defined herein, an "internal" nucleotide is one occurring at any position other than the 5' end or 3' end of nucleic acid molecule, polynucleotide or oligonucleotide. An internal nucleotide can be within a single-stranded molecule or within a strand of a duplex or double-stranded molecule. In one embodiment, the sense strand and/or antisense strand is modified by the substitution of at least one internal nucleotide. In another embodiment, the sense strand and/or antisense strand is modified by the substitution of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more internal nucleotides. In another embodiment, the sense strand and/or antisense strand is modified by the substitution of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the internal nucleotides. In yet another embodiment, the sense strand and/or antisense strand is modified by the substitution of all of the internal nucleotides.

[0065] In a preferred embodiment of the present invention the RNA molecule may contain at least one modified nucleotide analogue. The nucleotide analogues may be located at positions where the target-specific activity, e.g., the RNAi mediating activity is not substantially effected, e.g., in a

region at the 5'-end and/or the 3'-end of the RNA molecule. Particularly, the ends may be stabilized by incorporating modified nucleotide analogues.

[0066] Preferred nucleotide analogues include sugar- and/or backbone-modified ribonucleotides (i.e., include modifications to the phosphate-sugar backbone). For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. In preferred backbone-modified ribonucleotides the phosphoester group connecting to adjacent ribonucleotides is replaced by a modified group, e.g., of phosphothioate group. In preferred sugar-modified ribonucleotides, the 2' OH-group is replaced by a group selected from H, OR, R, halo, SH, SR, NH_2, NHR, NR_2 or ON, wherein R is C_1-C_6 alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I.

[0067] Also preferred are nucleobase-modified ribonucleotides, i.e., ribonucleotides, containing at least one non-naturally occurring nucleobase instead of a naturally occurring nucleobase. Bases may be modified to block the activity of adenosine deaminase. Exemplary modified nucleobases include, but are not limited to, uridine and/or cytidine modified at the 5-position, e.g., 5-(2-amino)propyl uridine, 5-bromo uridine; adenosine and/or guanosines modified at the 8 position, e.g., 8-bromo guanosine; deaza nucleotides, e.g., 7-deaza-adenosine; O- and N-alkylated nucleotides, e.g., N6-methyl adenosine are suitable. It should be noted that the above modifications may be combined.

4. RNA Synthesis

[0068] RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by in vitro enzymatic or organic synthesis. In one embodiment, an RNAi agent is prepared chemically. Methods of synthesizing RNA molecules are known in the art, in particular, the chemical synthesis methods as de scribed in Verma and Eckstein (1998) *Annul Rev. Biochem.* 67:99-134.

[0069] In another embodiment, a ss-siRNA is prepared enzymatically. For example, a ds-siRNA can be prepared by enzymatic processing of a long ds RNA having sufficient complementarity to the desired target mRNA. Processing of long ds RNA can be accomplished in vitro, for example, using appropriate cellular lysates and ds-siRNAs can be subsequently purified by gel electrophoresis or gel filtration. ds-siRNA can then be denatured according to art-recognized methodologies.

[0070] In an exemplary embodiment, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. Alternatively, the siRNA can also be prepared by enzymatic transcription from synthetic DNA templates or from DNA plasmids isolated from recombinant bacteria. Typically, phage RNA polymerases are used such as T7, T3 or SP6 RNA polymerase (Milligan and Uhlenbeck (1989) *Methods Enzymol.* 180:51-62). The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to inhibit annealing, and/or promote stabilization of the single strands.

[0071] In one embodiment, the single or double blunt-ended siRNAs are synthesized either in vivo, in situ, or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo or in situ, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the ss-siRNA. Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. A transgenic organism that expresses ss-siRNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

5. Selecting a Gene Target

[0072] In one embodiment, the target mRNA of the invention encodes the amino acid sequence of a cellular protein, e.g., a protein involved in cell growth or suppression, e.g., a nuclear, cytoplasmic, transmembrane, membrane-associated protein, or cellular ligand. In another embodiment, the target mRNA of the invention specifies the amino acid sequence of an extracellular protein (e.g., an extracellular matrix protein or secreted protein). Typical classes of proteins are listed for illustrative purposes.

[0073] Developmental proteins suitable for targeting according to the invention include e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors).

[0074] Oncogene-encoded proteins suitable for targeting according to the invention include, e.g., ABLI, BCLI, BCL2, BCL6, CBFA2, CBL, CSFIR, ERBA, ERBB, EBRB2, ETSI, ETSI, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCLI, MYCN, NRAS, PIM I, PML, RET, SRC, TALI, TCL3, and YES).

[0075] Tumor suppressor proteins suitable for targeting according to the invention include e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF I, NF2, RB I, TP53, and WTI).

[0076] Enzymatic proteins suitable for targeting according to the invention include, e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextriinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hernicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, xylanases, and telomerases.

[0077] In a preferred aspect of the invention, the target mRNA molecule of the invention specifies the amino acid sequence of a protein associated with a pathological condition. For example, the protein may be a pathogen-associated

protein (e.g., a viral protein involved in immunosuppression of the host, replication of the pathogen, transmission of the pathogen, or maintenance of the infection), or a host protein which facilitates entry of the pathogen into the host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of infection in the host, or assembly of the next generation of pathogen. Alternatively, the protein may be a tumor-associated protein or an autoimmune disease-associated protein.

[0078] By modulating the expression of the foregoing proteins, valuable information regarding the function of such proteins and therapeutic benefits which may be obtained from such modulation can be obtained.

6. Assay for Testing Engineered RNA Precursors

[0079] *Drosophila* embryo lysates can be used to determine if the engineered siRNAs of the invention, e.g., single or double blunt-ended siRNA duplexes (but also, e.g., expressed shRNAs) have their intended function (see also Examples 1-3). This lysate assay is described in Tuschl et al., 1999, supra, Zamore et al., 2000, supra, and Hutvdgner et al., *Science* 293, 834-838 (2001). These lysates recapitulate RNAi in vitro, thus permitting investigation into, e.g., which strand enters the complex, is assembled into RISC, and is used as a guide strand for target destruction. Briefly, the test siRNA is incubated with *Drosophila* embryo lysate for various times, then assayed for the production of the mature siRNA by primer extension or Northern hybridization. As in the in vivo setting, mature RNA accumulates in the cell-free reaction. Thus, an RNA corresponding to the proposed precursor can be shown to be converted into a siRNA duplex in the *Drosophila* embryo lysate.

[0080] Furthermore, an engineered RNA precursor can be functionally tested in the *Drosophila* embryo lysates. In this case, the engineered RNA precursor is incubated in the lysate in the presence of a 5' radiolabeled target mRNA in a standard in vitro RNAi reaction for various lengths of time. The target mRNA can be 5' radiolabeled using guanylyl transferase (as described in Tuschl et al., 1999, supra and references therein) or other suitable methods. The products of the in vitro reaction are then isolated and analyzed on a denaturing acrylamide or agarose gel to determine if the target mRNA has been cleaved in response to the presence of the engineered RNA precursor in the reaction. The extent and position of such cleavage of the mRNA target will indicate if the engineering of the precursor created a pre-siRNA capable of mediating sequence-specific RNAi.

7. Methods of Introducing RNAs, Vectors, and Host Cells

[0081] Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, inhibit annealing of single strands, stabilize the single strands, or other-wise increase inhibition of the target gene.

[0082] RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing a cell or organism in a solution containing the RNA. Vascular or extravascular circulation, the blood or lymph system, and the cerebrospinal fluid are sites where the RNA may be introduced.

[0083] The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals

[0084] Alternatively, vectors, e.g., transgenes encoding a siRNA of the invention, i.e., having at least one blunt end, can be engineered into a host cell or transgenic animal using art recognized techniques.

8. Methods of Treatment:

[0085] The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant or unwanted target gene expression or activity. It is understood that "treatment" or "treating" as used herein, is defined as the application or administration of a therapeutic agent (e.g., a RNAi agent or vector or transgene encoding same) to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease or disorder, a symptom of disease or disorder or a predisposition toward a disease or disorder, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease or disorder, the symptoms of the disease or disorder, or the predisposition toward disease.

9. Prophylactic Methods

[0086] In another aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant or unwanted target gene expression or activity, by administering to the subject a therapeutic agent (e.g., a RNAi agent or vector or transgene encoding same). Subjects at risk for a disease which is caused or contributed to by aberrant or unwanted target gene expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the target gene aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of target gene aberrancy, for example, a target gene, target gene agonist or target gene antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

10. Therapeutic Methods

[0087] In yet another aspect, the invention pertains to methods of modulating target gene expression, protein expression or activity for therapeutic purposes. Accordingly, in an exemplary embodiment, the modulatory method of the

invention involves contacting a cell capable of expressing target gene with a therapeutic agent (e.g., a RNAi agent or vector or transgene encoding same) that is specific for the target gene or protein (e.g., is specific for the mRNA encoded by said gene or specifying the amino acid sequence of said protein) such that expression or one or more of the activities of target protein is modulated. These modulatory methods can be performed in vitro (e.g., by culturing the cell with the agent) or, alternatively, in vivo (e.g., by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant or unwanted expression or activity of a target gene polypeptide or nucleic acid molecule. Inhibition of target gene activity is desirable in situations in which target gene is abnormally unregulated and/or in which decreased target gene activity is likely to have a beneficial effect.

11. Pharmacogenomics

[0088] The therapeutic agents (e.g., a RNAi agent or vector or transgene encoding same) of the invention can be administered to individuals to treat (prophylactically or therapeutically) disorders associated with aberrant or unwanted target gene activity. In conjunction with such treatment, pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, a physician or clinician may consider applying knowledge obtained in relevant pharmacogenomics studies in determining whether to administer a therapeutic agent as well as tailoring the dosage and/or therapeutic regimen of treatment with a therapeutic agent.

[0089] Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, for example, Eichelbaum, M. et al. (1996) *Clin. Exp. Pharmacol. Physiol.* 23(10-11): 983-985 and Linder, M. W. et al. (1997) *Clin. Chem.* 43(2):254-266

12. Pharmaceutical Compositions

[0090] The invention pertains to uses of the above-described agents for therapeutic treatments as described infra. Accordingly, the modulators of the present invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, antibody, or modulatory compound and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration.

[0091] The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

13. Knockout and/or Knockdown Cells or Organisms

[0092] A further preferred use for the RNAi agents of the present invention (or vectors or transgenes encoding same) is a functional analysis to be carried out in eukaryotic cells, or eukaryotic non-human organisms, preferably mammalian cells or organisms and most preferably human cells, e.g. cell lines such as HeLa or 293 or rodents, e.g. rats and mice. By administering a suitable RNAi agent which is sufficiently complementary to a target mRNA sequence to direct target-specific RNA interference, a specific knockout or knockdown phenotype can be obtained in a target cell, e.g. in cell culture or in a target organism.

[0093] Thus, a further subject matter of the invention is a eukaryotic cell or a eukaryotic non-human organism exhibiting a target gene-specific knockout or knockdown phenotype comprising a fully or at least partially deficient expression of at least one endogeneous target gene wherein said cell or organism is transfected with at least one vector comprising DNA encoding an RNAi agent capable of inhibiting the expression of the target gene. It should be noted that the present invention allows a target-specific knockout or knockdown of several different endogeneous genes due to the specificity of the RNAi agent.

[0094] Gene-specific knockout or knockdown phenotypes of cells or non-human organisms, particularly of human cells or non-human mammals may be used in analytic to procedures, e.g. in the functional and/or phenotypical analysis of complex physiological processes such as analysis of gene expression profiles and/or proteomes. Preferably the analysis is carried out by high throughput methods using oligonucleotide based chips.

14. Transgenic Organisms

[0095] Engineered RNA precursors of the invention can be expressed in transgenic animals. These animals represent a model system for the study of disorders that are caused by, or exacerbated by, overexpression or underexpression (as compared to wildtype or normal) of nucleic acids (and their encoded polypeptides) targeted for destruction by the RNAi agents, e.g., siRNAs and shRNAs, and for the development of therapeutic agents that modulate the expression or activity of nucleic acids or polypeptides targeted for destruction.

[0096] Transgenic animals can be farm animals (pigs, goats, sheep, cows, horses, rabbits, and the like), rodents (such as rats, guinea pigs, and mice), non-human primates (for example, baboons, monkeys, and chimpanzees), and domestic animals (for example, dogs and cats). Invertebrates such as *Caenorhabditis elegans* or *Drosophila* can be used as well as non-mammalian vertebrates such as fish (e.g., zebrafish) or birds (e.g., chickens).

[0097] Engineered RNA precursors with stems of 18 to 30 nucleotides in length are preferred for use in mammals, such as mice. A transgenic founder animal can be identified based upon the presence of a transgene that encodes the new RNA precursors in its genome, and/or expression of the transgene in tissues or cells of the animals, for example, using PCR or Northern analysis. Expression is confirmed by a decrease in the expression (RNA or protein) of the target sequence.

[0098] Methods for generating transgenic animals include introducing the transgene into the germ line of the animal. One method is by microinjection of a gene construct into the

pronucleus of an early stage embryo (e.g., before the four-cell stage; Wagner et al., 1981, Proc. Natl. Acad. Sci. USA 78:5016; Brinster et al., 1985, Proc. Natl. Acad. Sci. USA 82:4438). Alternatively, the transgene can be introduced into the pronucleus by retroviral infection. A detailed procedure for producing such transgenic mice has been described (see e.g., Hogan et al., *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1986); U.S. Pat. No. 5,175,383 (1992)). This procedure has also been adapted for other animal species (e.g., Hammer et al., 1985, *Nature* 315:680; Murray et al., 1989, *Reprod. Fert. Devl.* 1:147; Pursel et al., 1987, *Vet. Immunol. Histopath.* 17:303; Rexroad et al., 1990, *J. Reprod. Fert.* 41 (suppl): 1 19; Rexroad et al., 1989, *Molec. Reprod. Devl.* 1:164; Simons et al., 1988, *BioTechnology* 6:179; Vize et al., 1988, J. Cell. Sci. 90:295; and Wagner, 1989, J. Cell. Biochem. 13B (suppl): 164). Clones of the non-human transgenic animals described herein can be produced according to the methods described in Wilmut et al. ((1997) *Nature,* 385:810-813) and PCT publication Nos. WO 97/07668 and WO 97/07669.

15. Screening Assays

[0099] The methods of the invention are also suitable for use in methods to identify and/or characterize potential pharmacological agents, e.g. identifying new pharmacological agents from a collection of test substances and/or characterizing mechanisms of action and/or side effects of known pharmacological agents.

[0100] Thus, the present invention also relates to a system for identifying and/or characterizing pharmacological agents acting on at least one target protein comprising: (a) a eukaryotic cell or a eukaryotic non-human organism capable of expressing at least one endogeneous target gene coding for said so target protein, (b) at least one RNAi agent molecule capable of inhibiting the expression of said at least one endogeneous target gene, and (c) a test substance or a collection of test substances wherein pharmacological properties of said test substance or said collection are to be identified and/or characterized. Further, the system as described above preferably comprises: (d) at least one exogenous target nucleic acid coding for the target protein or a variant or mutated form of the target protein wherein said exogenous target nucleic acid differs from the endogeneous target gene on the nucleic acid level such that the expression of the exogenous target nucleic acid is substantially less inhibited by the RNAi agent than the expression of the endogeneous target gene.

[0101] The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K. S. (1997) *Anticancer Drug Des.* 12:145).

[0102] Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann et al. (1994). *J. Med. Chem.* 37:2678; Cho et al. (1993) *Science* 261:1303; Carrell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop et al. (1994) *J. Med. Chem.* 37:1233.

[0103] Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) Nature 364:555-556), bacteria (Ladner U.S. Pat. No. 5,223,409), spores (Ladner U.S. Pat. No. '409), plasmids (Cull et al. (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla et al. (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner supra.)).

[0104] In a preferred embodiment, the library is a natural product library, e.g., a library produced by a bacterial, fungal, or yeast culture. In another preferred embodiment, the library is a synthetic compound library.

[0105] This invention is further illustrated by the following examples which should not be construed as limiting.

EXEMPLIFICATION

[0106] Throughout the examples, the following materials and methods were used unless otherwise stated.

Materials and Methods

[0107] In general, the practice of the present invention employs, unless otherwise indicated, conventional techniques of nucleic acid chemistry, recombinant DNA technology, molecular biology, biochemistry, and cell and cell extract preparation. See, e.g., *DNA Cloning*, Vols. 1 and 2, (D. N. Glover, Ed. 1985); *Oligonucleotide Synthesis* (M. J. Gait, Ed. 1984); *Oxford Handbook of Nucleic Acid Structure*, Neidle, Ed., Oxford Univ Press (1999); *RNA Interference: The Nuts & Bolts of siRNA Technology*, by D. Engelke, DNA Press, (2003); *Gene Silencing by RNA Interference: Technology and Application*, by M. Sohail, CRC Press (2004); Sambrook, Fritsch and Maniatis, *Molecular Cloning*: Cold Spring Harbor Laboratory Press (1989); and Current Protocols in Molecular Biology, eds. Ausubel et al., John Wiley & Sons (1992). See also PCT/US03/24768 (Attorney Docket No. UMY-033PC); U.S. Ser. No. 60/475,331 (Attorney Docket No. UMY-066-1); U.S. Ser. No. 60/507,928, (Attorney Docket No. UMY-066-2); and U.S. Ser. No. 60/475,386 (Attorney Docket No. UMY-050-1), of which all are incorporated in their entireties by reference herein.

siRNA Preparation

[0108] Synthetic RNAs (Dharmacon) were deprotected according to the manufacturer's protocol. siRNA strands were annealed (Elbashir et al., *Genes Dev* 15, 188-200 (2001) and used at 50 nM final concentration unless otherwise noted. siRNA single strands were phosphorylated with polynucleotide kinase (New England Biolabs) and 1 mM ATP according to the manufacturer's directions and used at 500 nM final concentration.

Sense and Anti-Sense Target Preparation

[0109] Target RNAs were transcribed with recombinant, histidine-tagged, T7 RNA Polymerase from PCR products

as described (Nykänen et al., 2001, supra; Hutvágner and Zamore, *Science* 297, 2056-2060 (2002), except for sense sod1 mRNA, which was transcribed from a plasmid template (Crow et al., *J Neurochem* 69, 1936-1944 (1997)) linearized with Bam HI. PCR templates for htt sense and antisense and sod1 antisense target RNAs were generated by amplifying 0.1 ng/ml (final concentration) plasmid template encoding htt or sod1 cDNA using the following primer pairs: htt sense target, 5'-GCG TAA TAC GAC TCA CTA TAG GAA CAG TAT GTC TCA GAC ATC-3' and 5'-UUCG AAG UAU UCC GCG UAC GU-3'; htt antisense target, 5'-GCG TAA TAC GAC TCA CTA TAG GAC AAG CCT AAT TAG TGATGC-3' and 5'-GAA CAG TAT GTC TCA GAC ATC-3'; sod1 antisense target, 5'-GCG TAA TAC GAC TCA CTA TAG GGC TTT GTT AGC AGC CGG AT-3' and 5'-GGG AGA CCA CAA CGG TTT CCC-3'.

RISC Extract Preparation

[0110] *Drosophila* embryo lysate preparation, in vitro RNAi reactions, and cap-labeling of target RNAs using guanylyl transferase were carried out as previously described (Tuschl et al., 1999, supra; Zamore et al., 2000, supra). Target RNAs were used at ~5 nM concentration to ensure that reactions occurred under single-turnover conditions. Target cleavage under these conditions was proportionate to siRNA concentrations. Cleavage products of RNAi reactions were analyzed by electrophoresis on 5% or 8% denaturing acrylamide gels. 5' end labeling and determination of siRNA unwinding status were according to Nykänen et al. (Nykänen et al., 2001, supra) except that unlabeled competitor RNA was used at 100-fold molar excess. Gels were dried, then exposed to image plates (Fuji), which were scanned with a Fuji FLA-5000 phosphorimager. Images were analyzed using Image Reader FLA-5000 version 1.0 (Fuji) and Image Gauge version 3.45 or 4.1 (Fuji). Data analysis was performed using Excel (Microsoft) and IgorPro 5.0 (Wavemetrics).

Example 1

Functionally Asymmetric siRNA Duplexes Having a Single Blunt End

[0111] The following example describes methods for constructing single blunt-ended siRNA duplexes capable of selectively entering a RISC-mediated RNAi pathway and selectively cleaving a test target for destruction.

[0112] Briefly, to assess quantitatively if the two strands of an siRNA duplex having a single 5' or 3' blunt end are equally competent to direct RNAi, the individual rates of sense and antisense target cleavage for a single blunt-ended siRNA duplex directed against the SOD1 target gene were examined (**FIG. 4**). The relevant portions of the sense and antisense target RNA sequences are shown in **FIG. 4** and in schematic form in **FIG. 1** (see lower panel). The single blunt-ended siRNA duplex effectively silences SOD1 expression in *Drosophila* extracts when having a weakened 5' end (i.e., "frayed end") (compare 4 with 1 in **FIGS. 1 and 4**).

[0113] Accordingly, these results indicate that 1) a single blunt end siRNA is functional and 2) that weakening the 5' antisense base pair interaction with the 3' sense strand dramatically increases entry of the antisense strand into the complex and subsequent gene knockdown activity.

Example 2

Functionally Asymmetric siRNA Duplexes Having a Double Blunt Ends

[0114] The following example describes methods for constructing double blunt-ended siRNA duplexes capable of selectively entering a RISC-mediated RNAi pathway and selectively cleaving a test target for destruction.

[0115] Briefly, to assess quantitatively if the two strands of an double blunt-ended siRNA duplex are equally competent to direct RNAi, the individual rates of sense and antisense target cleavage for a single blunt-ended siRNA duplex directed against the SOD1 target gene were examined (**FIG. 5**). The relevant portions of the sense and antisense target RNA sequences are shown in **FIG. 3** and in schematic form in **FIG. 2** (see lower panel). The double blunt-ended siRNA duplexes effectively silence SOD1 expression in *Drosophila* extracts and this activity is increased in the when having a weakened 5' end (i.e., "frayed end") (compare 4 with 1 in **FIGS. 1 and 4**).

[0116] Accordingly, these results indicate that 1) double blunt-end siRNA molecules are functional and 2) that weakening the 5' antisense base pair interaction with the 3' sense strand or the 5' sense base pair interaction with the 3' antisense strand modulates the entry of the antisense and sense strand into the complex and subsequent gene knockdown activity (see **FIGS. 2 and 5**).

Example 3

Single and Double Blunt-Ended siRNA Strand Contribution in RISC Assembly

[0117] The following example describes methods for determining RISC-mediated selectivity regarding the single and double blunt-ended siRNAs of the invention.

[0118] To identify the source of asymmetry in the function of such an single or double blunt-ended siRNA duplex, the unwinding of the two siRNA strands when the duplex is incubated in a standard in vitro RNAi reaction is measured. This assay has been observed to determine accurately the fraction of siRNA that is unwound in an ATP-dependent step in the RNAi pathway and that no functional RISC is assembled in the absence of ATP (Nykänen et al., 2001). Other observations have noted that siRNA unwinding correlates with capacity of an siRNA to function in target cleavage (Nykänen et al., 2001, supra; Martinez et al., *Cell* 110, 563-574 (2002)), demonstrating that siRNA duplex unwinding is required to assemble a RISC competent to base pair with its target RNA.

[0119] Accordingly, the accumulation of single stranded siRNA against a test gene such as luciferase after 1 hour incubation in an in vitro RNAi reaction in the absence of target RNA is measured. After one hour of incubation with *Drosophila* embryo lysate in a standard RNAi reaction, the antisense strand of the luciferase siRNA is converted to single-strand. In control experiments, single-stranded RNA is assayed without incubation in lysate. Since the production of single-stranded antisense siRNA must be accompanied by an equal amount of single-stranded sense siRNA, the missing sense-strand is calculated to have been destroyed after unwinding.

[0120] To establish that the observed asymmetry in the accumulation of the two single-strands is not an artifact of our unwinding assay, an independent method for measuring the fraction of siRNA present as single-strands in protein-RNA complexes is performed. In this assay, double-stranded siRNA is incubated with *Drosophila* embryo lysate in a standard RNAi reaction for 1 h, then a 31 nt 2'-O-methyl RNA oligonucleotide containing a 21 nt sequence complementary to the radiolabeled siRNA strand is added. 2'-O-methyl oligonucleotides are not cleaved by the RNAi machinery, but can bind stably to complementary siRNA within the RISC. To allow recovery of RISC, the 2'-O-methyl oligonucleotide is tethered to a magnetic bead via a biotin-streptavidin linkage. After washing away unbound RNA and protein, the amount of radioactive siRNA bound to the bead is measured. The assay is performed with separate siRNA duplexes in which either the sense or the antisense strand is 5'-^{32}P-radiolabeled. Capture of ^{32}P-siRNA is observed when the 2'-O-methyl oligonucleotide contained a 21-nt region complementary to the radiolabeled siRNA strand, but not when an unrelated oligonucleotide is used.

[0121] Thus, the above assay captures all RISC activity directed by the siRNA strand complementary to the tethered oligonucleotide, demonstrating that it measures siRNA present in the lysate as single-strand complexed with RISC proteins.

[0122] Accordingly, this assay can determine the contribution each strand from a single or double blunt-ended siRNA of the invention makes to RISC assembly.

EQUIVALENTS

[0123] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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<400> SEQUENCE: 22

gagacuuggg caaugugaa 19

<210> SEQ ID NO 23
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 23

uucacauugc ccaagucuct t 21

<210> SEQ ID NO 24
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 24

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 25
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 25

gagacuuggg caaugugaa 19

<210> SEQ ID NO 26
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: inosine

<400> SEQUENCE: 26

ucacauugc ccaagucuct t 21

<210> SEQ ID NO 27

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<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 27

augaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 28
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 28

gagacuuggg caaugugaa 19

<210> SEQ ID NO 29
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 29

gucacauugc ccaagucuat t 21

<210> SEQ ID NO 30
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 30

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 31
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 31

gagacuuggg caaugugact t 21

<210> SEQ ID NO 32
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 32

gucacauugc ccaagucuc 19

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<210> SEQ ID NO 33
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 33

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 34
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 34

uagacuuggg caaugugact t 21

<210> SEQ ID NO 35
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 35

gucacauugc ccaagucuc 19

<210> SEQ ID NO 36
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 36

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 37
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: inosine

<400> SEQUENCE: 37

nagacuuggg caaugugact t 21

<210> SEQ ID NO 38
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic

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siRNA Molecule

<400> SEQUENCE: 38

gucacauugc ccaagucuc 19

<210> SEQ ID NO 39
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 39

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 40
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 40

gagauugggc aaugugaatt 20

<210> SEQ ID NO 41
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 41

gucacaauug cccaagucuc 20

<210> SEQ ID NO 42
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 42

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 43
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 43

gagacuuggg caaugugaut t 21

<210> SEQ ID NO 44
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 44

gucacauugc ccaagucuc 19

<210> SEQ ID NO 45
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 45

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 46
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 46

gagacuuggg caaugugact t 21

<210> SEQ ID NO 47
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 47

gucacauugc ccaagucua 19

<210> SEQ ID NO 48
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 48

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 49
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 49

uagacuuggg caaugugact t 21

<210> SEQ ID NO 50
<211> LENGTH: 19

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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 50

gucacauugc ccaagucua 19

<210> SEQ ID NO 51
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 51

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 52
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: inosine

<400> SEQUENCE: 52

nagacuuggg caaugugact t 21

<210> SEQ ID NO 53
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 53

gucacauugc ccaagucua 19

<210> SEQ ID NO 54
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 54

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 55
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 55

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gagacuuggg caaugugaat t 21

<210> SEQ ID NO 56
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic siRNA Molecule

<400> SEQUENCE: 56

gucacauugc ccaagucua 19

<210> SEQ ID NO 57
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 57

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 58
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule:
Synthetic siRNA
Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
siRNA Molecule

<400> SEQUENCE: 58

gagacuuggg caaugugaut t 21

<210> SEQ ID NO 59
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic siRNA Molecule

<400> SEQUENCE: 59

gucacauugc ccaagucua 19

<210> SEQ ID NO 60
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 60

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 61
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
siRNA Molecule

<400> SEQUENCE: 61

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gagacuuggg caaugugac 19

<210> SEQ ID NO 62
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic siRNA Molecule

<400> SEQUENCE: 62

gucacauugc ccaagucuc 19

<210> SEQ ID NO 63
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 63

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 64
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
siRNA Molecule

<400> SEQUENCE: 64

gagacuuggg caaugugac 19

<210> SEQ ID NO 65
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic siRNA Molecule

<400> SEQUENCE: 65

gucacauugc ccaagucua 19

<210> SEQ ID NO 66
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 66

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 67
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
siRNA Molecule

<400> SEQUENCE: 67

gagacuuggg caaugugaa 19

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<210> SEQ ID NO 68
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 68

gucacauugc ccaagucuc 19

<210> SEQ ID NO 69
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 69

gaugaagaga ggcauguugg agacuugggc aaugugacug cugacaaaga uggu 54

<210> SEQ ID NO 70
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 70

gagacuuggg caaugugaa 19

<210> SEQ ID NO 71
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 71

gucacauugc ccaagucua 19

<210> SEQ ID NO 72
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 72

gaugaagaga ggcauguugg agacuugggc aaugugagug cugacaaaga uggu 54

<210> SEQ ID NO 73
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 73

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

<210> SEQ ID NO 74
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

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<400> SEQUENCE: 74

gagacuuggg caaugugac 19

<210> SEQ ID NO 75
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Mol
<400> SEQUENCE: 75

gucacauugc ccaagucuct t 21

<210> SEQ ID NO 76
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 76

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

<210> SEQ ID NO 77
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 77

gagacuuggg caaugugac 19

<210> SEQ ID NO 78
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 78

uucacauugc ccaagucuct t 21

<210> SEQ ID NO 79
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 79

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

<210> SEQ ID NO 80
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic

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siRNA Molecule

<400> SEQUENCE: 80

gagacuuggg caaugugac 19

<210> SEQ ID NO 81
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: inosine

<400> SEQUENCE: 81

nucacauugc ccaagucuct t 21

<210> SEQ ID NO 82
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 82

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

<210> SEQ ID NO 83
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 83

gagacuuggg caaugugac 19

<210> SEQ ID NO 84
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 84

gucacauugc ccaagucuat t 21

<210> SEQ ID NO 85
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 85

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

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<210> SEQ ID NO 86
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 86

gagacuuggg caaugugaa 19

<210> SEQ ID NO 87
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 87

gucacauugc ccaagucuct t 21

<210> SEQ ID NO 88
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 88

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

<210> SEQ ID NO 89
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 89

gagacuuggg caaugugaa 19

<210> SEQ ID NO 90
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 90

uucacauugc ccaagucuct t 21

<210> SEQ ID NO 91
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 91

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

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<210> SEQ ID NO 92
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 92

gagacuuggg caaugugaa 19

<210> SEQ ID NO 93
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
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<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: inosine

<400> SEQUENCE: 93

nucacauugc ccaagucuct t 21

<210> SEQ ID NO 94
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 94

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

<210> SEQ ID NO 95
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 95

gagacuuggg caaugugaa 19

<210> SEQ ID NO 96
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 96

gucacauugc ccaagucuat t 21

<210> SEQ ID NO 97
<211> LENGTH: 54

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<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 97

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

<210> SEQ ID NO 98
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 98

gagacuuggg caaugugact t 21

<210> SEQ ID NO 99
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 99

gucacauugc ccaagucuc 19

<210> SEQ ID NO 100
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 100

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

<210> SEQ ID NO 101
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 101

uagacuuggg caaugugact t 21

<210> SEQ ID NO 102
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic siRNA Molecule

<400> SEQUENCE: 102

gucacauugc ccaagucuc 19

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<210> SEQ ID NO 103
<211> LENGTH: 54
<212> TYPE: RNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 103

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

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nagacuuggg caaugugact t 21

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<400> SEQUENCE: 105

gucacauugc ccaagucuc 19

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<400> SEQUENCE: 106

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<400> SEQUENCE: 107

gagacuuggg caaugugaat t 21

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siRNA Molecule

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gucacauugc ccaagucuc 19

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<400> SEQUENCE: 109

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<210> SEQ ID NO 110
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gagacuuggg caaugugaut t 21

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<400> SEQUENCE: 111

gucacauugc ccaagucuc 19

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gagacuuggg caaugugact t 21

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gucacauugc ccaagucua 19

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<400> SEQUENCE: 115

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uagacuuggg caaugugact t 21

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gucacauugc ccaagucua 19

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<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
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<400> SEQUENCE: 119

nagacuuggg caaugugact t 21

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gucacauugc ccaagucua 19

<210> SEQ ID NO 121
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<400> SEQUENCE: 121

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<210> SEQ ID NO 122
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gagacuuggg caaugugaat t 21

<210> SEQ ID NO 123
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<400> SEQUENCE: 123

gucacauugc ccaagucua 19

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<400> SEQUENCE: 124

accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

<210> SEQ ID NO 125
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<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule: Synthetic siRNA Molecule
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<400> SEQUENCE: 125

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gagacuuggg caaugugaut t 21

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<213> ORGANISM: Artificial Sequence
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gucacauugc ccaagucua 19

<210> SEQ ID NO 127
<211> LENGTH: 54
<212> TYPE: RNA
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<400> SEQUENCE: 127

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gagacuuggg caaugugac 19

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accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

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gagacuuggg caaugugac 19

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gucacauugc ccaagucua 19

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gagacuuggg caaugugaa 19

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gucacauugc ccaagucuc 19

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accaucuuug ucagcaguca cauugcccaa gucuccaaca ugccucucuu cauc 54

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<400> SEQUENCE: 137

gagacuuggg caaugugaa 19

<210> SEQ ID NO 138
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<220> FEATURE:
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<400> SEQUENCE: 138

gucacauugc ccaagucua 19

1. A method of enhancing the ability of a first strand of a RNAi agent to act as a guide strand in mediating RNAi, the RNAi agent derived from an RNA duplex having at least one blunt end, comprising lessening the base pair strength between the 5' end of the first strand and the 3' end of a second strand of the duplex as compared to the base pair strength between the 3' end of the first strand and the 5' end of the second strand.

2. A method of enhancing the efficacy of a siRNA duplex having at least one blunt end, the siRNA duplex comprising a sense and an antisense strand, comprising lessening the base pair strength between the antisense strand 5' end (AS 5') and the sense strand 3' end (S 3') as compared to the base pair strength between the antisense strand 3' end (AS 3') and the sense strand 5' end (S '5), such that efficacy is enhanced.

3. A method of enhancing the ability of a first strand of a RNAi agent to act as a guide strand in mediating RNAi, the RNAi agent derived from an RNA duplex having 5' and 3' blunt ends, comprising lessening the base pair strength between the 5' end of the first strand and the 3' end of a second strand of the duplex as compared to the base pair strength between the 3' end of the first strand and the 5' end of the second strand.

4. A method of enhancing the efficacy of a siRNA duplex having 5' and 3' blunt ends, the siRNA duplex comprising a sense and an antisense strand, comprising lessening the base pair strength between the antisense strand 5' end (AS 5') and the sense strand 3' end (S 3') as compared to the base pair strength between the antisense strand 3' end (AS 3') and the sense strand 5' end (S '5), such that efficacy is enhanced.

5. A method of promoting entry of a desired strand of an siRNA duplex having at least one blunt end into a RISC complex, comprising enhancing the asymmetry of the siRNA duplex, such that entry of the desired strand is promoted.

6. The method of claim 5, wherein the siRNA duplex has 5' and 3' blunt ends.

7. The method of claim 5, wherein asymmetry is enhanced by lessening the base pair strength between the 5' end of the desired strand and the 3' end of a complementary strand of the duplex as compared to the base pair strength between the 3' end of the desired strand and the 5' end of the complementary strand.

8. The method of any one of claims **1**, **2**, **3**, or **4**, wherein the base-pair strength is less due to fewer G:C base pairs between the 5' end of the first or antisense strand and the 3' end of the second or sense strand than between the 3' end of the first or antisense strand and the 5' end of the second or sense strand.

9. The method of any one of claims **1**, **2**, **3**, or **4**, wherein the base pair strength is less due to at least one mismatched base pair between the 5' end of the first or antisense strand and the 3' end of the second or sense strand.

10. The method of claim 9, wherein the mismatched base pair is selected from the group consisting of G:A, C:A, C:U, G:G, A:A, C:C, U:U, I:A, I:U, and I:C.

11. The method of any one of claims **1**, **2**, **3**, or **4**, wherein the base pair strength is less due to at least one wobble base pair between the 5' end of the first or antisense strand and the 3' end of the second or sense strand.

12. The method of claim 11, wherein the wobble base pair is G:U.

13. The method of claim 11, wherein the wobble base pair is G:T.

14. The method of any one of claims **1**, **2**, **3**, or **4**, wherein the base pair strength is less due to:

(a) at least one mismatched base pair between the 5' end of the first or antisense strand and the 3' end of the second or sense strand; and

(b) at least one wobble base pair between the 5' end of the first or antisense strand and the 3' end of the second or sense strand.

15. The method of claim 14, wherein the mismatched base pair is selected from the group consisting of G:A, C:A, C:U, G:G, A:A, C:C and U:U.

16. The method of claim 14, wherein the mismatched base pair is selected from the group consisting of G:A, C:A, C:T, G:G, A:A, C:C and U:T.

17. The method of claim 14, wherein the wobble base pair is G:U.

18. The method of claim 14, wherein the wobble base pair is G:T.

19. The method of any one of claims **1**, **2**, **3**, or **4**, wherein the base pair strength is less due to at least one base pair comprising a rare nucleotide.

20. The method of claim 19, wherein the modified nucleotide is selected from the group consisting of 2-amino-G, 2-amino-A, 2,6-diamino-G, and 2,6-diamino-A.

21. The method of claim 1, wherein the RNAi agent is a siRNA duplex.

22. The method of any one of claims **1**, **2**, **3**, or **4**, wherein the RNAi agent or siRNA duplex is chemically synthesized.

23. The method of any one of claims **1**, **2**, **3**, or **4**, wherein the RNAi agent or siRNA duplex is enzymatically synthesized.

24. The method of any one of claims **1**, **2**, **3**, or **4**, wherein the RNAi agent or siRNA duplex is derived from an engineered precursor.

25. A method of enhancing silencing of a target mRNA, comprising contacting a cell having an RNAi pathway with the RNAi agent or siRNA duplex of claims **1**, **2**, **3**, or **4** under conditions such that silencing is enhanced.

26. A method of enhancing silencing of a target mRNA in a subject, comprising administering to the subject a phar-

maceutical composition comprising the RNAi agent or siRNA duplex of claims **1**, **2**, **3**, or **4** such that silencing is enhanced.

27. A method of decreasing silencing of an inadvertent target mRNA by a dsRNAi agent, the dsRNAi agent comprising a sense strand, an antisense strand, and having at least one blunt end comprising:

(a) detecting a significant degree of complementarity between the sense strand and the inadvertent target; and

(b) enhancing the base pair strength between the 5' end of the sense strand and the 3' end of the antisense strand relative to the base pair strength between the 3' end of the sense strand and the 5' end of the antisense strand;

such that silencing of the inadvertent target mRNA is decreased.

28. The method of claim 27, wherein silencing of the inadvertent target mRNA is decreased relative to silencing of a desired target mRNA.

29. An RNAi agent or siRNA duplex having 5' and 3' blunt ends comprising a sense strand and an antisense strand, wherein the base pair strength between the antisense strand 3' end (AS 3') and the sense strand 5' end (S 5') is less than the base pair strength between the antisense strand 5' end (AS 5') and the sense strand 3' end (S '3), such that the antisense strand preferentially guides cleavage of a target mRNA.

30. The siRNA duplex of claim 29, wherein the base-pair strength is less due to fewer G:C base pairs between the AS 5' and the S 3' than between the AS 3' and the S 5'.

31. The siRNA duplex of claim 29, wherein the base pair strength is less due to at least one mismatched base pair between the AS 5' and the S 3'.

32. The siRNA duplex of claim 31, wherein the mismatched base pair is selected from the group consisting of G:A, C:A, C:T, U:T, C:U, G:G, A:A, C:C, U:U, I:A, I:U, and I:C.

33. The siRNA duplex of claim 29, wherein the base pair strength is less due to at least one wobble base pair between the AS 5' and the S 3'.

34. The siRNA duplex of claim 29, wherein the wobble base pair is G:U.

35. The siRNA duplex of claim 29, wherein the wobble base pair is G:T.

36. The siRNA duplex of claim 29, wherein the base pair strength is less due to at least one base pair comprising a modified nucleotide.

37. The siRNA duplex of claim 36, wherein the modified nucleotide is selected from the group consisting of 2-amino-G, 2-amino-A, 2,6-diamino-G, and 2,6-diamino-A.

38. A composition comprising the RNAi agent or siRNA duplex of claim 29, formulated to facilitate entry of the RNAi agent or siRNA duplex into a cell.

39. A pharmaceutical composition comprising the RNAi agent or siRNA duplex of claim 38.

40. An engineered pre-miRNA comprising the RNAi agent or siRNA duplex of claim 38.

41. A vector encoding the pre-miRNA of claim 40.

42. A pre-miRNA comprising the pre-miRNA of claim 41.

43. A vector encoding the pre-miRNA of claim 42.

44. A small hairpin RNA (shRNA) comprising nucleotide sequence identical to the sense and antisense strand of the siRNA duplex of claim 29.

45. The shRNA of claim 44, wherein the nucleotide sequence identical to the sense strand is upstream of the nucleotide sequence identical to the antisense strand.

46. The shRNA of claim 44, wherein the nucleotide sequence identical to the antisense strand is upstream of the nucleotide sequence identical to the sense strand.

47. A vector encoding the shRNA of any one of claims **44-46**.

48. A cell comprising the vector of any one of claims **41**, **43**, or **47**.

49. The cell of claim 48, which is a mammalian cell.

50. The cell of claim 48, which is a human cell.

51. A transgene encoding the shRNA of any one of claims **44-46**.

* * * * *