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**Original Article**

# *In Vivo* Activity and Duration of Short Interfering RNAs Containing a Synthetic 5′-Phosphate

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Endogenous and exogenous short interfering RNAs (siRNAs) require a 5′-phosphate for loading into Ago2 and cleavage of the target mRNA. We applied a synthetic 5′-phosphate to siRNA guide strands to evaluate if phosphorylation *in vivo* is rate limiting for maximal siRNA knockdown and duration. We report, for the first time, an *in vivo* evaluation of siRNAs with a synthetic 5′-phosphate compared to their unphosphorylated versions. siRNAs that contained a 5′-phosphate had the same activity *in vivo* compared with unphosphorylated siRNAs, indicating phosphorylation of an siRNA is not a rate limiting step *in vivo*.

## Introduction

Double stranded RNAs, such as small interfering RNAs (siRNAs), induce degradation of sequence specific homologous mRNA via RNA interference (RNAi), a mechanism of posttranslational gene silencing (Dorsett and Tuschl, 2004). Specific gene silencing has the potential to not only identify new drug targets, but also to be developed into a therapy whereby a synthetic siRNA can be used to enter the endogenous RNAi pathway and eliminate a gene of interest. This allows for targeting of genes relevant in a disease pathway that may be outside of the small molecule druggable genome (Corey, 2007; de Fougerolles et al., 2007).

siRNAs are RNA duplexes of 19–21 nucleotides with 2 nucleotide 3′ overhangs (Elbashir et al., 2001a; Elbashir et al., 2001b; Harborth et al., 2001). Naturally occurring mammalian siRNAs and micro RNAs (miRNAs) have 5′-phosphate (5′-P) groups and 3′-hydroxyl (3′-OH) groups resulting from the cleavage of their longer precursors' phosphodiester bonds (Carthew and Sontheimer, 2009; Kawamata et al., 2011). However, in order for synthetic siRNAs to be effective upon entering the cell, the siRNA must become phosphorylated on the 5′ end by Clp1 kinase and be incorporated into the endogenous RNA-induced silencing complex (RISC), which consists of Argonaute 2 (Ago2), Dicer, and TRBP (Elbashir et al., 2001b; Doi et al., 2003; Rivas et al., 2005; Sen and Blau, 2006; Weitzer and Martinez, 2007). During this assembly, one of the two strands of the siRNA, known as the passenger strand, is cleaved, whereby the other strand, referred to as the guide strand, binds to Ago2 (Martinez et al., 2002; Matranga et al., 2005; Rand et al., 2005). The guide strand is bound to the MID domain of Ago2, which recognizes the 5′-P on the guide strand and anchors the 5′ end during loading (Nykanen et al., 2001; Ma et al., 2005; Frank et al., 2011). The guide strand in the RISC complex associates with the complementary messenger RNA (mRNA) strand, cleaves the mRNA, and regulates gene expression. The fate of the 5′-P after cleavage is unknown. While the 5′-P was continuously removed and replenished in *Drosophila* studies, when the same studies were done in mammalian systems, there appeared to be no phosphatase available and the 5′-P remained on the siRNA (Boutla et al., 2001; Nykanen et al., 2001; Schwarz et al., 2002; Weitzer and Martinez, 2007).

Previously, we demonstrated that 5′-phosphorylation was necessary for activity of siRNAs *in vivo* (Kenski et al., 2009). Using unlocked nucleic acid (UNA) modifications, we showed siRNAs modified with UNAs at position 1 or 2 were unable to be phosphorylated by Clp1 kinase and exhibited decreased binding toAgo2, reduced cleavage activity by Ago2 and less mRNA degradation *in vitro* and *in vivo*. Cell based mRNA degradation, Ago2 binding, and cleavage activity of the UNA modified siRNA at position 1 was successfully recovered by the chemical addition of a 5′-P on the UNA modified siRNA. The 5′-P was also able to recover the activity of the position 1 UNA modified siRNA *in vivo*. Since the UNA modification at position 1 did not impair Ago2 functioning when a 5′-P was present, we concluded that it was the lack of phosphorylation, not the modification, that prevented efficient activity of the siRNA.

Other studies using siRNAs containing different 2′-modifications at position 1 or modifications of the 5′-OH group have demonstrated the importance of the 5′-P group. Modifications such as 2′-altriol modifications or a 2′-F-4′-thioate at position 1 on siRNAs had attenuated potency that was recovered after synthetically applying a 5′-P (Fisher et al., 2007; Watts et al., 2007). When the 5′-OH was directly

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modified, siRNA activity was also abrogated. An amino group with a 3-carbon linker was used to replace the 5′-OH on an siRNA, which blocked phosphorylation by Clp1 and decreased activity (Chiu and Rana, 2003). Another study using 5′-O-methyl modified siRNAs also prevented 5′-phosphorylation from occurring and consequent guide strand loading into RISC for mRNA degradation (Chen et al., 2008). These studies emphasize the critical role of 5′-phosphorylation on the ability of a siRNA to function in the cell and that 5′-phosphorylation of an siRNA is necessary and may be a rate limiting step for RISC loading and cleavage.

Understanding the efficiency of phosphorylation of an siRNA and how that relates to maximal target mRNA degradation and duration of activity *in vivo* is critical for therapeutic optimization of an siRNA. In order to test if phosphorylation is the rate limiting step for maximal siRNA knockdown and duration we evaluated, *in vivo*, siRNAs that contain ribonucleotides at positions 1 and 2 of their guide strand and are therefore not hindered in their ability to be phosphorylated by Clp1. We then applied a synthetic phosphate to the 5′ ends of the siRNA guide strands and compared them against siRNAs with a 5′-OH that could be naturally phosphorylated by Clp1. We observed that siRNAs containing a synthetic 5′-P had the same mRNA degradation and duration *in vivo* compared to the unphosphorylated siRNA with a natural 5′-OH, demonstrating phosphorylation of an siRNA is not a rate limiting step for *in vivo* activity.

## Materials and Methods

### *Oligo synthesis and sequences*

Chemically modified siRNAs contained ribo (r), deoxy (d), fluoro (flu), or methoxy (ome) 2′ modifications as described previously (Zou et al., 2008). 5′-P was applied at the 5′ end of the guide strand when indicated using standard phosphoramidite chemistry. siRNAs were synthesized as described previously (Wincott, 2001). The siRNA sequences and modification are as follows:

Luciferase(80) [Luc(80)] unmodified siRNA
Passenger: 5′-iB;rA;rU;rA;rA;rG;rG;rC;rU;rA;rU;rG;rA;rA;rG;rA;rG;rA;rU;rA;dT;dT;iB-3′
Guide: 5′-rU;rA;rU;rC;rU;rC;rU;rU;rC;rA;rU;rA;rG;rC;rC;rU;rU;rA;rU;dT;dT-3′
Luc(80) modified siRNA
Passenger: 5′-iB;dA;fluU;dA;dA;dG;dG;fluC;fluU;dA;fluU;dG;dA;dA;dG;dA;dG;dA;fluU;dA;dT;dT;iB-3′
Guide: 5′-rU;rA;rU;fluC;fluU;fluC;fluU;fluU;fluC;omeA;fluU;omeA;omeG;fluC;fluC;fluU;fluU;omeA;fluU;omeU;omeU-3′
ApoB(9514) unmodified siRNA
Passenger: 5′-iB;rC;rU;rU;rU;rA;rA;rC;rA;rA;rU;rU;rC;rC;rU;rG;rA;rA;rA;rU;omeU;omeU;iB-3′
Guide: 5′-rA;rU;rU;rU;rC;rA;rG;rG;rA;rA;rU;rU;rG;rU;rU;rA;rA;rA;rG;omeU;omeU-3′
ApoB(9514) modified siRNA Passenger: 5′-iB;omeC;omeU;omeU;omeU;fluA;fluA;omeC;fluA;fluA;omeU;omeU;omeC;omeC;omeU;fluG;fluA;fluA;fluA;omeU;omeUs;omeU;iB-3′
Guide: 5′-rAs;rUs;rUs;omeU;omeC;fluA;fluG;fluG;fluA;fluA;omeU;omeU;fluG;fluU;omeU;fluA;fluA;fluA;fluG;omeUs;omeU-3′
ApoB(10162) unmodified siRNA
Passenger: 5′-iB;rC;rA;rA;rG;rU;rG;rU;rC;rA;rU;rC;rA;rC;rA;rC;rU;rG;rA;rA;omeU;omeU;iB-3′
Guide: 5′-rU;rU;rC;rA;rG;rU;rG;rU;rG;rA;rU;rG;rA;rC;rA;rC;rU;rU;rG;omeU;omeU-3′
PHD2(196) unmodified siRNA
Passenger: 5′-iB;rC;rA;rU;rU;rG;rA;rA;rC;rC;rC;rA;rA;rA;rU;rU;rU;rG;rA;rU;omeU;omeU;iB-3′
Guide: 5′-rA;rU;rC;rA;rA;rA;rU;rU;rU;rG;rG;rG;rU;rU;rC;rA;rA;rU;rG;omeU;omeU-3′
PHD2(384) unmodified siRNA
Passenger: 5′-iB;rC;rA;rG;rU;rC;rA;rG;rC;rA;rA;rA;rG;rA;rC;rG;rU;rC;rU;rA;omeU;omeU;iB-3′
Guide: 5′-rU;rA;rG;rA;rC;rG;rU;rC;rU;rU;rU;rG;rC;rU;rG;rA;rC;rU;rG;omeU;omeU-3′
SSB(869) unmodified siRNA
Passenger: 5′-iB;rA;rA;rA;rU;rC;rA;rU;rG;rG;rU;rG;rA;rA;rA;rU;rA;rA;rA;rA;omeU;omeU;iB-3′
Guide: 5′-rU;rU;rU;rU;rA;rU;rU;rU;rC;rA;rC;rC;rA;rU;rG;rA;rU;rU;rU;omeU;omeU-3′

### *Transfection and quantitative polymerase chain reaction*

Transfection and quantitative polymerase chain reaction (qPCR) for *in vitro* and *in vivo* studies were done as described previously (Kenski et al., 2009).

### *siRNA formulation*

Lipid nanoparticles were made using the cationic lipid 2-{4-[(3b)-cholest-5-en-3-yloxy]-octyl}-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Merck and Co., Inc.), cholesterol (Northern Lipids), and monomethoxypolyethyleneglycol-1,2-dimyristoylglycerol (NOF Corporation) at a 60:38:2 M ratio, respectively.

### *In vivo*

C57BL/6 male mice 20–23g were purchased from Taconic Farms, Inc. Mice were injected intravenously with 200 μL of 3 mg/kg siRNA formulated in a lipid nanoparticle. Four mice per group were sacrificed at indicated time points following siRNA injection. Livers were harvested and processed to assess target mRNA levels by qPCR as described above.

### *Luciferase mouse model*

The luciferase mouse model, imaging measurements, and analysis were done as described previously (Wei et al., 2010).

## Results

### *5′-Phosphate does not alter siRNA potency*

We first evaluated if siRNA *in vitro* potency was effected with and without a 5′-P. Six different double stranded 21-mer siRNA sequences were evaluated *in vitro*: ApoB(9514), ApoB(10162), PHD2(196), PHD2(384), SSB(869), and Luc(80). Positions 1–19 of both strands were ribonucleotides, and the overhangs at positions 20 and 21 contained 2′-O-methoxy nucleotides except for Luc(80), which contained 2′-deoxyribonucleotide thymidines (Fig. 1). The passenger strands contained inverted abasics at the 5′ and 3′ ends to block loading into Ago2 (Campochiaro, 2006).

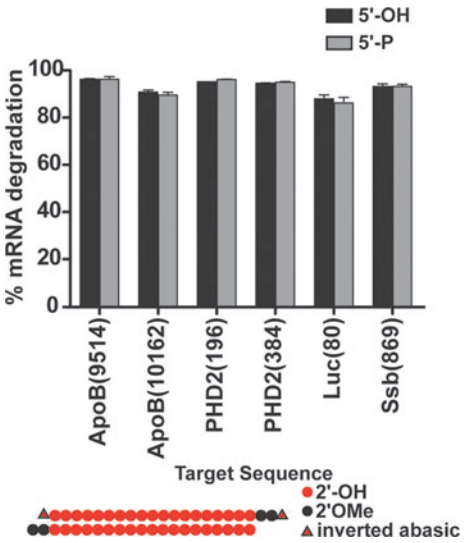


**FIG. 1.** Synthetic 5′-phosphate (5′-P) does not alter *in vitro* messenger RNA (mRNA) degradation of siRNAs. Six short interfering RNA (siRNA) sequences containing complementary all ribose guide and passenger strands and either a 5′-hydroxyl (5′-OH) (black bar) or a 5′-P (gray bar) at the 5′-termini were evaluated for maximal mRNA degradation. No change was seen for any of the sequences with or without 5′-P.

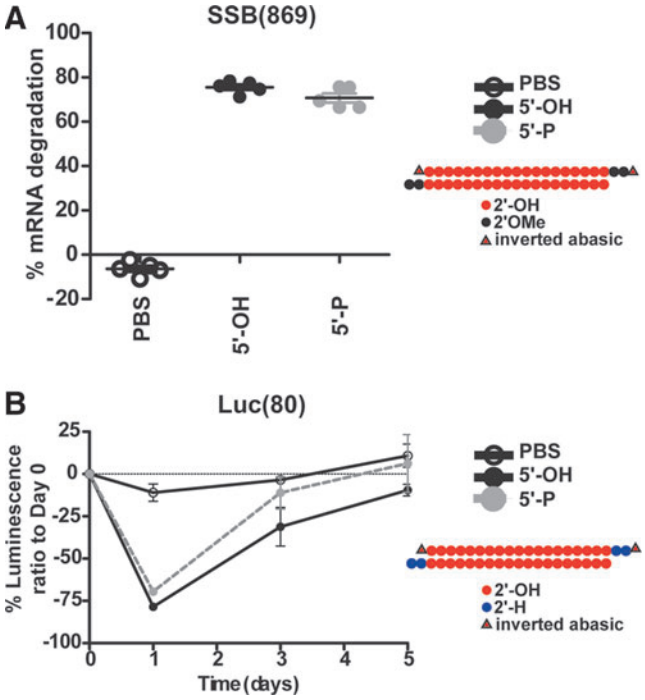


**FIG. 2.** Synthetic 5′-P does not alter *in vivo* mRNA degradation of unmodified siRNAs. SSB(869) (**A**) and Luc(80) (**B**) containing complementary all ribose guide and passenger strands and either a 5′-OH (black line) or a 5′-P (gray line) at the 5′-termini were evaluated in mice for mRNA degradation over time. No change was seen for any of the sequences with or without 5′-P. Data are presented as the mean and standard error of the mean (SEM).

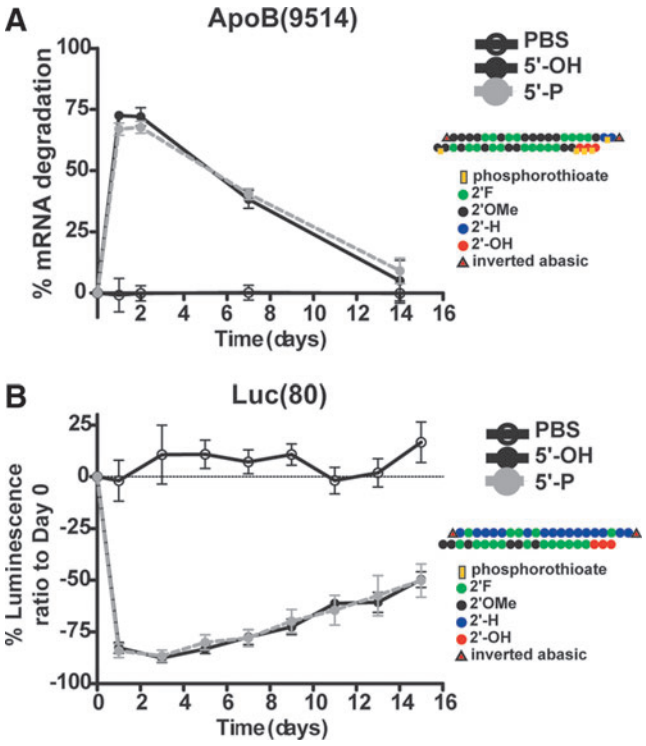


**FIG. 3.** Synthetic 5′-P does not alter the *in vivo* mRNA degradation of modified siRNAs. ApoB(9514) (**A**) and Luc(80) (**B**) containing 2′-deoxy (2′-H), 2′-fluoro (2′-F) and 2′-methoxy (2′-OMe) modification on their guide and passenger strands and either a 5′-OH (black line) or a 5′-P (gray line) at the 5′-termini were evaluated in mice for mRNA degradation over time. No change was seen for any of the sequences with or without 5′-P. Data are presented as the mean and SEM.

All siRNAs demonstrated target specific mRNA degradation in cell based assays as detected by qPCR. Hepa 1-6 cells were transfected with either an unmodified siRNA containing a natural 5′-OH that can be phosphorylated by Clp1 in cells (Schwarz et al., 2002) or a synthetic 5′-P on the guide strand. These siRNAs were tested for maximal mRNA degradation at 10 nM (Fig. 1A) and half maximal effective concentration (EC50) values (Table 1). For all sequences tested, unmodified siRNA with a 5′-P on the guide strand exhibited no change in maximal mRNA degradation or dose responses *in vitro* compared to the unphosphorylated version.

Table 1. *In Vitro* Half Maximal Effective Concentrations and Silencing RNA Sequences

| | *Sequence* | *EC50 (nM)* | |
|---|---|---|---|
| | Passenger 5′-3′ | 5′-OH | 5′-P |
| ApoB(9514) | CUUUAACAAUUCCUGAAAU | 0.3 | 0.44 |
| ApoB(10162) | CAAGUGUCAUCACACUGAA | 0.5 | 0.51 |
| PHD2(196) | CAUUGAACCCAAAUUUGAU | 0.05 | 0.05 |
| PHD2(384) | CAGUCAGCAAAGACGUCUA | 0.13 | 0.14 |
| Luc(80) | AUAAGGCUAUGAAGAGAUA | 0.19 | 0.6 |
| SSB(869) | AAAUCAUGGUGAAAUAAA | 0.02 | 0.02 |

5′-OH, 5′-hydroyl; 5′-P 5′-phosphate.

### *5′-Phosphate does not change activity in vivo*

In order to assess the if synthetic 5′-terminal phosphorylation of an siRNA improves maximal mRNA degradation or duration *in vivo*, SSB(869) and Luc(80) siRNAs containing unmodified ribonucleotides with or without a 5′-P were formulated into lipid nanoparticles, which localize to the liver, and delivered by intravenous tail injection into mice. Livers were harvested for SSB(869) 24 hours after injection, and mRNA levels were assessed by qPCR (Fig. 2A). For SSB(869), unmodified siRNA with a natural 5′-OH had 75% mRNA degradation 24 hours after injection. The same SSB(869) siRNA that contained a synthetically 5′-P had 70% mRNA degradation, which was equivalent to the unphosphorylated version at 24 hours.

For Luc(80), mice that constitutively expressed Luc from the Rosa promoter in their livers were used and imaged for luciferase activity every other day after siRNA injection (Wei et al., 2010). The unmodified siRNAs contained either a 5′-OH or a synthetic 5′-P. The 5′-OH siRNA had a similar mRNA degradation at day 1 as the 5′-P siRNA, with 78% and 70% mRNA degradation, respectively. The *in vivo* duration of the 5′-OH and 5′-P containing Luc(80) siRNAs were also not different from each other (Fig. 2B).

Since some siRNAs made only of ribonucleotides can be limited in their *in vivo* duration and potency and may have immunostimulatory properties, we designed heavily modified siRNA sequences containing 2′-methoxy (2′-OMe), 2′-deoxy (2′-H), and 2′-fluoro (2′-F) nucleotides. 2′-OMe modifications on the guide strand can reduce off target effects and, when on either the passenger or guide strand, reduce immune responses (Jackson et al., 2006; Robbins et al., 2007). 2′-F or 2′-H modifications can increase potency, serum stability and half-life while reducing immunostimulatory responses (Chiu and Rana, 2003; Layzer et al., 2004).

An ApoB(9514) siRNA was designed with a passenger strand containing 2′-OMe pyrimidines and 2′-F purines in positions 1–19 (Fig. 3A). The passenger strand overhangs were 2′-H with a phosphorothioate linkage and the 5′ and 3′ ends were capped with inverted abasics to prevent loading. Positions 1–3 on the guide strand were ribonucleotides with phosphorothioate linkages in order to maintain Clp1 recognition (Kenski et al., 2009). Positions 4–19 contained 2′-OMe pyrimidines and 2′-F purines. The guide strand overhangs were 2′-OMe with a phosphorothioate linkage for serum stability. *In vivo*, these modified siRNAs with or without a 5′-P did not significantly differ in maximal mRNA degradation at day 2, with 72% and 68%, respectively. The duration of activity was similar at day 14 for both siRNAs as well.

A different modification pattern was designed for Luc(80) in order to maintain maximal activity (Fig. 3B). The passenger strand contained 2′-F pyrimidines, 2′-H purines and overhangs, and the 5′ and 3′ ends were capped with inverted abasics. Positions 1–3 on the guide strand were ribonucleotides, as was designed for ApoB(9514). Positions 4–19 on the guide strand contained 2′-F pyrimidines and 2′-OMe purines. The guide strand overhangs were 2′-OMe. The maximal mRNA degradation *in vivo* for the Luc(80) siRNAs with or without a 5′-P was identical with 87% mRNA degradation observed for both siRNAs at day 3. The duration of activity was equivalent at day 14 for both siRNAs.

## Discussion

This is the first *in vivo* evaluation of siRNAs with synthetic 5′-P. When 5′-P was synthetically applied to multiple siRNA sequences there was no difference *in vivo* of maximal mRNA degradation or duration of activity compared to the same unphosphorylated siRNA. These observations held true for unmodified and modified versions of siRNAs. This indicates while 5′P is needed for efficient Ago2 loading, it may not be the rate limiting step for achieving ideal *in vivo* results. Optimization of siRNAs for therapeutic uses will require further identification of the steps in the endogenous RISC machinery that lead to target degradation and development of modifications that will facilitate the most efficient use of siRNAs with this complex.

Phosphorylation of the 5′-termius of the guide strand is a crucial step for RNAi silencing to occur (Boutla et al., 2001; Schwarz et al., 2002). In the Ago2 crystal structure, the 5′-phosphorylated nucleotide at position 1 is located in the MID domain, with the 5′ end of the siRNA fitting into a basic pocket with the 5′ base stacking on the aromatic ring of a tyrosine which helps to further stabilize binding (Yuan et al., 2006; Wang et al., 2008). Ago2 interdomain interactions were shown to be critical in siRNA anchoring whereby a 5′-phosphorylated RNA fragment could not bind to the MID domain alone, but also required the PIWI domain of Ago2. In addition, the seed region of the siRNA, which is comprised of guide strand positions 2–8, has multiple interactions with the MID and PIWI domains of Ago2 in order to stabilize it in the correct conformation for hybridization to the target mRNA (Djuranovic et al., 2010). These studies suggest that the 5′-P, position 1 nucleotide and seed region of the siRNA all play a role in anchoring the siRNA to Ago2 for effective target mRNA degradation (Boland et al., 2011; Kawamata et al., 2011). The 5′-P on the siRNA may therefore serve as a reference point from which the cleavage site is measured. Further structural investigation of these regions of Ago2, and how siRNAs are conformationally controlled by them, will be useful for optimization of siRNAs.

The 5′-P is critical for siRNA strand selection, anchoring of the siRNA to Ago2, and as the start of a ruler for correct cleavage of the target mRNA. Yet, very few derivatives have been made to take advantage of the role of the 5′-P in an siRNA for maximal therapeutic activity. One area that has benefitted has been the in the development of single stranded siRNAs (ssRNA) that require a 5′-P to be active (Schwarz et al., 2002). In using ssRNA, the cost of goods and negative phosphodiester backbone charges are cut in half while delivery may be made easier. Recently, a modified ssRNA that included a 5′ vinyl phosphonate was evaluated and shown to improve the stability of the ssRNA molecule. Additional modification of the 5′-P will be necessary to improve the therapeutic value of ssRNA or duplex siRNA.

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## Author Disclosure Statement

No competing financial interests exist.

## References


BOLAND, A., HUNTZINGER, E., SCHMIDT, S., IZAURRALDE, E. and WEICHENRIEDER, O. (2011). Crystal structure of the MID-PIWI lobe of a eukaryotic Argonaute protein. Proc. Natl. Acad. Sci. U. S. A. 108, 10466–10471.

BOUTLA, A., DELIDAKIS, C., LIVADARAS, I., TSAGRIS, M., and TABLER, M. (2001). Short 5′-phosphorylated double-stranded RNAs induce RNA interference in *Drosophila*. Curr. Biol. 11, 1776–1780.

CAMPOCHIARO, P. A. (2006). Potential applications for RNAi to probe pathogenesis and develop new treatments for ocular disorders. Gene Ther. 13, 559–562.

CARTHEW, R. W. and SONTHEIMER, E. J. (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell 136, 642–55.

CHEN, P. Y., WEINMANN, L., GAIDATZIS, D., PEI, Y., ZAVOLAN, M., TUSCHL, T., and MEISTER, G. (2008). Strand-specific 5′-O-methylation of siRNA duplexes controls guide strand selection and targeting specificity. RNA 14, 263–274.

CHIU, Y. L. and RANA, T. M. (2003). siRNA function in RNAi: a chemical modification analysis. RNA 9, 1034–1048.

COREY, D. R. (2007). RNA learns from antisense. Nat. Chem. Biol. 3, 8–11.

DE FOUGEROLLES, A., VORNLOCHER, H. P., MARAGANORE, J., and LIEBERMAN, J. (2007). Interfering with disease: a progress report on siRNA-based therapeutics. Nat. Rev. Drug Discov. 6, 443–453.

DJURANOVIC, S., ZINCHENKO, M. K., HUR, J. K., NAHVI, A., BRUNELLE, J. L., ROGERS, E. J., and GREEN, R. (2010). Allosteric regulation of Argonaute proteins by miRNAs. Nat. Struct. Mol. Biol. 17, 144–150.

DOI, N., ZENNO, S., UEDA, R., OHKI-HAMAZAKI, H., UI-TEI, K., and SAIGO, K. (2003). Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. Curr. Biol. 13, 41–46.

DORSETT, Y. and TUSCHL, T. (2004). siRNAs: applications in functional genomics and potential as therapeutics. Nat. Rev. Drug Discov. 3, 318–329.

ELBASHIR, S. M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001a). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411,** 494–8.

ELBASHIR, S. M., LENDECKEL, W., and TUSCHL, T. (2001b). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **15,** 188–200.

FISHER, M., ABRAMOV, M., VAN AERSCHOT, A., XU, D., JULIANO, R. L. and HERDEWIJN, P. (2007). Inhibition of MDR1 expression with altritol-modified siRNAs. Nucleic Acids Res. **35,** 1064–1074.

FRANK, F., SONENBERG, N., and NAGAR, B. (2011). Structural basis for 5′-nucleotide base-specific recognition of guide RNA by human AGO2. Nature **465,** 818–822.

HARBORTH, J., ELBASHIR, S. M., BECHERT, K., TUSCHL, T., and WEBER, K. (2001). Identification of essential genes in cultured mammalian cells using small interfering RNAs. J. Cell Sci. **114,** 4557–4565.

JACKSON, A. L., BURCHARD, J., LEAKE, D., REYNOLDS, A., SCHELTER, J., GUO, J., JOHNSON, J. M., LIM, L., KARPILOW, J., NICHOLS, K., et al. (2006). Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA **12,** 1197–1205.

KAWAMATA, T., YODA, M., and TOMARI, Y. (2011). Multilayer checkpoints for microRNA authenticity during RISC assembly. EMBO Rep. **12,** 944–949.

KENSKI, D. M., COOPER, A. J., LI, J. J., WILLINGHAM, A. T., HARINGSMA, H. J., YOUNG, T. A., KUKLIN, N. A., JONES, J. J., CANCILLA, M. T., MCMASTERS, D. R., et al. (2009). Analysis of acyclic nucleoside modifications in siRNAs finds sensitivity at position 1 that is restored by 5′-terminal phosphorylation both *in vitro* and *in vivo*. Nucleic Acids Res. **38,** 660–671.

LAYZER, J. M., MCCAFFREY, A. P., TANNER, A. K., HUANG, Z., KAY, M. A., and SULLENGER, B. A. (2004). *In vivo* activity of nuclease-resistant siRNAs. RNA 10, 766–771.

MA, J. B., YUAN, Y. R., MEISTER, G., PEI, Y., TUSCHL, T., and PATEL, D. J. (2005). Structural basis for 5′-end-specific recognition of guide RNA by the *A. fulgidus* Piwi protein. Nature **434,** 666–670.

MARTINEZ, J., PATKANIOWSKA, A., URLAUB, H., LUHRMANN, R., and TUSCHL, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell **110,** 563–574.

MATRANGA, C., TOMARI, Y., SHIN, C., BARTEL, D. P., and ZAMORE, P. D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell **123,** 607–620.

NYKANEN, A., HALEY, B., and ZAMORE, P. D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309–321.

RAND, T. A., PETERSEN, S., DU, F., and WANG, X. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell **123,** 621–629.

RIVAS, F. V., TOLIA, N. H., SONG, J. J., ARAGON, J. P., LIU, J., HANNON, G. J., and JOSHUA-TOR, L. (2005). Purified Argonaute2 and an siRNA form recombinant human RISC. Nat. Struct. Mol. Biol. **12,** 340–349.

ROBBINS, M., JUDGE, A., LIANG, L., MCCLINTOCK, K., YAWORSKI, E., and MACLACHLAN, I. (2007). 2′-O-methyl-modified RNAs act as TLR7 antagonists. Mol. Ther. **15,** 1663–1669.

SCHWARZ, D. S., HUTVAGNER, G., HALEY, B., and ZAMORE, P. D. (2002). Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. Mol. Cell **10,** 537–548.

SEN, G. L. and BLAU, H. M. (2006). A brief history of RNAi: the silence of the genes. Faseb. J. **20,** 1293–1299.

WANG, Y., SHENG, G., JURANEK, S., TUSCHL, T., and PATEL, D. J. (2008). Structure of the guide-strand-containing argonaute silencing complex. Nature **456,** 209–213.

WATTS, J. K., CHOUBDAR, N., SADALAPURE, K., ROBERT, F., WAHBA, A. S., PELLETIER, J., PINTO, B. M., and DAMHA, M. J. (2007). 2′-fluoro-4′-thioarabino-modified oligonucleotides: conformational switches linked to siRNA activity. Nucleic Acids Res. 35, 1441–1451.

WEI, J., JONES, J., KANG, J., CARD, A., KRIMM, M., HANCOCK, P., PEI, Y., ASON, B., PAYSON, E., DUBININA, N., et al. (2010). RNA-induced silencing complex-bound small interfering RNA is a determinant of RNA interference-mediated gene silencing in mice. Mol. Pharmacol. **79,** 953–963.

WEITZER, S. and MARTINEZ, J. (2007). The human RNA kinase hClp1 is active on 3′ transfer RNA exons and short interfering RNAs. Nature **447,** 222–226.

WINCOTT, F. E. (2001). Strategies for oligoribonucleotide synthesis according to the phosphoramidite method. Curr. Protoc. Nucleic Acid Chem. **3,** 3.5.1–3.5.12.

YUAN, Y. R., PEI, Y., CHEN, H. Y., TUSCHL, T., and PATEL, D. J. (2006). A potential protein-RNA recognition event along the RISC-loading pathway from the structure of *A. aeolicus* Argonaute with externally bound siRNA. Structure **14,** 1557–1565.

ZOU, Y., TILLER, P., CHEN, I. W., BEVERLY, M., and HOCHMAN, J. (2008). Metabolite identification of small interfering RNA duplex by high-resolution accurate mass spectrometry. Rapid Commun. Mass Spectrom. **22,** 1871–1881.

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