

## RESEARCH ARTICLE

### 3'-Poly(A) tail enhances siRNA activity against exogenous reporter genes in MCF-7 cells

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#### ABSTRACT

RNA interference (RNAi) is a powerful tool in the study of gene function. We added poly(A) tails to the 3' ends of siRNA antisense strands by *in vitro* transcription, and investigated the silencing effects of poly(A)-tailed siRNAs on enhanced green fluorescence protein (EGFP) and red fluorescence protein (RFP) genes. The results of this study showed that siRNAs with single-stranded 3'-poly(A) tails at antisense strands had noticeably stronger silencing effect on exogenous reporter genes than their corresponding parental forms. The enhanced silencing effect appears to be related to the length of poly(A) but was non siRNA sequence-specific. Furthermore, our results demonstrate that weakly-activated PKR and reduced stability of mRNAs of exogenous reporter genes *in vivo* may be the possible mechanisms of this non-specific enhanced silencing effect. Our findings are likely to be of value in designing siRNAs with enhanced activity.

**KEYWORDS:** *siRNA, poly(A) tail, non-specific, enhanced gene silencing*

#### INTRODUCTION

RNA interference (RNAi) is the process by which dsRNA silences gene expression, either by inducing the sequence-specific degradation of complementary mRNA or by inhibiting translation. RNAi was initially defined in the worm *Caenorhabditis elegans* by Fire et al (Fire et al, 1998), and we now know it is conserved from fungi to mammals. The RNAi pathway has many cellular functions, including protecting cells against viruses and repetitive DNA elements, directing the assembly of heterochromatin, and regulating cellular development, growth, differentiation, and apoptosis. A simplified model for RNAi pathway involves two steps. In the first step, long double-stranded RNA or microRNA primary transcripts are converted to segments of short interfering RNA (siRNA), which are 21-23 bp long, by the RNase III-like protein Dicer (Bernstein et al, 2001), leaving 5'-phosphate 3'-hydroxy termini and 2 nt 3' overhangs. In the second step, these products are incorporated into the RNA-induced silencing complex (RISC) (Hammond, 2005). The siRNA is unwound in a strand-specific manner during

RISC formation. Gene silencing results from translational inhibition or degradation of target mRNA, depending upon the siRNA/mRNA duplex complementarity.

Long dsRNAs (>30 bp) cannot be used in mammals with provoking the antiviral/interferon response (Williams, 1999). It was shown that siRNAs with 21-23 bp which can be synthesized chemically or transcribed *in vitro*, could bypass the interferon pathway (Elbashir et al, 2001; Paddison et al, 2002). However, recent reports suggest some otherwise (Sledz et al, 2003; Bridge et al, 2003). Adding dTdT or UU to the 3' end of siRNAs may improve silencing efficiency based the finding that the PAZ domain can interact with the 3' end of single-stranded RNA. The PAZ domain, which is found in Dicer family proteins and Argonaute proteins, can bind 3' overhangs of siRNAs, possibly anchoring the 3' end of guide RNA within the silencing effector complex (Lingel et al, 2003; Song et al, 2003; Yan et al, 2003). However, dTdT and UU are not the only 3' overhangs of siRNAs, and the effects of alternative 3' overhang sequences on the efficiency and specificity of RNAi are unclear. In the present study, we

investigated the effects on the activity of siRNAs when a single-stranded poly(A) was added to the 3' end of the antisense strand.

## MATERIALS AND METHODS

### Synthesis and purification of RNA

DNA oligonucleotides with T7 primers were annealed to produce dsDNA fragments. All RNAs were synthesized by *in vitro* transcription, purified using denaturing polyacrylamide gel (Bevilacqua and Cech, 1996), and treated with calf intestinal alkaline phosphatase (Promega) as described previously (Kim et al, 2004). The ssRNAs were annealed to form dsRNAs in annealing buffer (50 mM Tris, pH 7.5, 100 mM NaCl in DEPC-treated water). The RNAs used in these studies were as follows:-

#### EGFP siRNA:

5'-CAAGCUGACCCUGAAGUUCAU-3'  
5'-AACUUCAGGGUCAGCUUGCCG-3'

#### EGFP siRNA-18A (*gsi18A*):

5'-CAAGCUGACCCUGAAGUUCAU-3'  
5'-ACUUCAGGGUCAGCUUGCCGA<sub>18</sub>-3'

#### EGFP siRNA-36A (*gsi36A*):

5'-CAAGCUGACCCUGAAGUUCAU-3'  
5'-AACUUCAGGGUCAGCUUGCCGA<sub>36</sub>-3'

#### EGFP siRNA-60A (*gsi60A*):

5'-CAAGCUGACCCUGAAGUUCAU-3'  
5'-AACUUCAGGGUCAGCUUGCCGA<sub>60</sub>-3'

#### EGFP siRNA-60U (*gsi60U*):

5'-CAAGCUGACCCUGAAGUUCAU-3'  
5'-AACUUCAGGGUCAGCUUGCCGU<sub>60</sub>-3'

#### EGFP siRNA-60N (*gsi60N, N-negative*):

5'-CAAGCUGACCCUGAAGUUCAU-3'  
5'-AACUUCAGGGUCAGCUUGCCGGUAACGCCAG  
GGUUUCCGUCCUUGUCGAUACUGGCCUAAAUC  
CACUGUGAUUAUCUAAU-3'

#### Negative control siRNA (*nsi*):

5'-UUCUCCGAACGUGUCACGUUG-3'  
5'-ACGUGACACGUUCGGAGAAUG-3'

#### Negative siRNA -18A (*nsi18A*):

5'-UUCUCCGAACGUGUCACGUUG-3'  
5'-ACGUGACACGUUCGGAGAAUG A<sub>18</sub>-3'

#### Negative control siRNA -36A (*nsi36A*):

5'-UUCUCCGAACGUGUCACGUUG-3'  
5'-ACGUGACACGUUCGGAGAAUG A<sub>36</sub>-3'

#### Negative control siRNA -60A (*nsi60A*):

5'-UUCUCCGAACGUGUCACGUUG-3'  
5'-ACGUGACACGUUCGGAGAAUG A<sub>60</sub>-3'

#### RFP siRNA (*rsi*):

5'-AGUCCAGUACGGCUCCAAGG-3'  
5'-UUGGAGCCGUACUGGAACUUG-3'

#### RFP siRNA-18A (*rsi18A*):

5'-AGUCCAGUACGGCUCCAAGG-3'  
5'-UUGGAGCCGUACUGGAACUUG A<sub>18</sub>-3'

#### RFP siRNA-36A (*rsi36A*):

5'-AGUCCAGUACGGCUCCAAGG-3'  
5'-UUGGAGCCGUACUGGAACUUG A<sub>36</sub>-3'

#### RFP siRNA-60A (*rsi60A*):

5'-AGUCCAGUACGGCUCCAAGG-3'  
5'-UUGGAGCCGUACUGGAACUUG A<sub>60</sub>-3'

10A: 5'-A<sub>10</sub>-3'

20A: 5'-A<sub>20</sub>-3'

30A: 5'-A<sub>30</sub>-3'

40A: 5'-A<sub>40</sub>-3'

50A: 5'-A<sub>50</sub>-3'

60A: 5'-A<sub>60</sub>-3'

60U: 5'-U<sub>60</sub>-3'

60N (N is negative control): 5'-GGTAACGCCAGGGTTT  
TCCGTCCTTTGTCGATACTGGCTAAATCCACTGTG  
ATATCTAAT-3'

### Transfection and silencing assay

All transfections were carried out using lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). MCF-7 cells (90% confluent) were transfected in 24-well plates. A reporter gene vector (200 ng) and 40-nM RNA were co-transfected, and the ratio of lipofectamine:DNA+RNA (μl:μg) was 2:1. Reporter gene expression was visualized by fluorescence microscopy 48 hr after transfection. The reporter gene expression vectors pEGFP-N2 (EGFP) and pdsRED2-N1 (RFP) were purchased from Clontech.

### RT-PCR analysis of mRNA and DNA

Total RNA and DNA were isolated from cells using TRIzol® reagent (Invitrogen). cDNAs were generated using M-MLV Reverse Transcriptase (Promega) and oligo(dT)<sub>15</sub> primer (Promega) according to the manufacturer's instructions, typically using 500 ng of total RNA per reaction. The polymerase chain reaction (PCR) was performed on 2 μl cDNA in 50 μl of final volume. Samples were analyzed by electrophoresis on 1.5% (w/v) agarose gel and visualized by ethidium bromide staining. The results were normalized with β-actin internal control. The various primers are below:

#### The EGFP primers:

5'-GCAAGCTGACCCTGAAGTTCATC-3' (forward)  
5'-TCACCTTGATGCCGTTCTTCTG-3' (reverse)

#### The RFP primers:

5'-CGAGGGCTTCAAGTGGGA-3' (forward)  
5'-CTACAGGAACAGGTGGTGGC-3' (reverse)

#### The β-actin primers:

5'-CATCTCTGCTCGAAGTCCA-3' (forward)  
5'-ATCATGTTTGAGACCTTCAACA-3' (reverse).

### Bacterial expression and purification of GST-PKR

The PKR expression plasmid, pEGST-PKR/λPP, was a gift from Dr Takayasu Date at Kanazawa Medical University.

GST-PKR was expressed in *Escherichia coli* BL21 and purified as described previously (Matsui et al, 2001).

#### ***In vitro* PKR kinase assay**

GST-PKR (2 µg) was dephosphorylated with 400 U of λ-PPase (Upstate, Charlottesville, VA) at 37°C for 30 min, and the reaction was quenched with 2 mM sodium orthovanadate (Sigma). A 10 µl mixture containing 0.5 µg of treated GST-PKR, 20 µM ATP, 10 µCi of [ $\gamma$ -<sup>32</sup>P]ATP, the designated concentrations of RNAs and buffer (50 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 50 mM KCl, pH 7.5) was incubated for 30 min at 23°C. Reactions were quenched using SDS sample buffer. Phosphorylated proteins were separated via SDS/PAGE and visualized by autoradiography.

#### **Cell lysis and immunoblotting**

Cells were washed with PBS (25°C) and then lysed over 20 min in cold lysis buffer (50 mM Tris-HCl; pH 7.4; 1% (v/v) NP-40; 0.25% (w/v) sodium deoxycholate; 150 mM NaCl; 1 mM each EDTA, PMSF, Na<sub>3</sub>VO<sub>4</sub>, and NaF; 1 µg/ml each aprotinin, leupeptin, and pepstatin). Extracts were clarified by centrifugation (10,000 × g) for 20 min at 4°C. Protein concentrations were determined using the Bradford assay. Proteins were separated using 10% (w/v) SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in a solution of TBS containing 5% (w/v) non-fat dry milk and 0.05% (v/v) Tween-20 (TBST-MLK) for 30 min at 25°C with constant agitation, incubated with primary antibodies per the manufacturer's instructions for 2 hr at 25°C, washed with TBST, and incubated for 1 hr with a goat anti-rabbit HRP-conjugated IgG (1:5000 dilution). After three additional washes, enhanced chemiluminescence (ECL) was performed according to manufacturer's protocol (Amersham). The anti-phospho-PKR (Thr446, rabbit polyclonal IgG) was purchased from Upstate, and the polyclonal antibodies against β-actin were obtained from Santa Cruz.

#### **Preparation of <sup>32</sup>P-labeled mRNA fragments**

A 262-base fragment of EGFP mRNA (bases 46-308) was prepared by *in vitro* transcription from DNA templates containing T7 RNA polymerase promoter. The DNA templates were prepared by PCR amplification using the following primers:

5'-GCATGCTAATACGACTCACTATAGGGCACATC  
GCTCAGACACCA-3' (forward)  
5'-GAGCACAGGGTACTTTATTGA-3' (reverse)

Poly(A) tail was added to the 3' end of the siRNAs using poly(A) polymerase (TaKaRa) as per the manufacturer's protocol. The 5' end of poly(A) mRNA was labeled using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP (MEGALABEL™ Kit, TaKaRa).

#### ***In vitro* mRNA degradation**

*In vitro* mRNA degradation assay was modified according to the method described previously (Ross and Kobs, 1986; Tuschl et al, 1999). Briefly, MCF-7 cells were transfected with different tiny RNAs (40 nM). After 12 hr, cellular extracts from were prepared by homogenization in low-

salt buffer (10 mM Tris-HCl pH 7.6, 1 mM magnesium acetate, 1.5 mM potassium acetate, 2 mM dithiothreitol, 0.1 mM PMSF, 1 µg/ml each aprotinin, leupeptin, pepstatin). mRNA was allowed to decay at 37°C for various lengths of time. Reactions took place in 20 µl volumes containing 0.2 A260 U of cellular extracts, 10 nM tiny RNAs [siRNA, siRNA-poly(A) or poly(A)], and 50 pM mRNA (containing equal amounts of 5'-<sup>32</sup>P-labeled mRNA) in buffer [10 mM Tris-HCl pH 7.6, 2 mM magnesium acetate, 1 mM potassium acetate, 2 mM dithiothreitol, 10 mM creatine phosphate, 0.1 U creatine phosphokinase, 1 mM ATP, 0.4 mM GTP, 0.1 mM spermine, and 2 U of RNasin (Promega)]. At each time point, RNA was extracted and processed via electrophoresis in 4% (w/v) polyacrylamide 7 M urea gels (Bernstein et al, 1989).

#### **Measurement of mRNA stability *in vivo***

The EGFP reporter gene was transfected in MCF-7 cells for 12 hr. Cells were then transfected with groups of RNA (40 nM), and Actinomycin D (ActD, 10 µg/ml) was added during RNA treatment as described previously (Jing et al, 2005). Total RNA was isolated at different time points after RNA treatment. The stability of EGFP mRNA was determined via quantitative PCR using an Applied Biosystems Prism 7000, SYBR Green PCR MasterMix (QiaGen, TIANGEN), and specific primers (listed below) to detect EGFP and GAPDH. Target gene expression was normalized using a standard curve, and data were analyzed using Applied Biosystems Prism software and the  $\Delta\Delta C_t$  method (Vandesompele et al, 2002). Each sample was analyzed in duplicate.

#### *The EGFP primers (for qPCR):*

5'-CGACGGCAACTACAAGAC-3' (forward)  
5'-TAGTTGTACTCCAGCTTGTGC-3' (reverse)

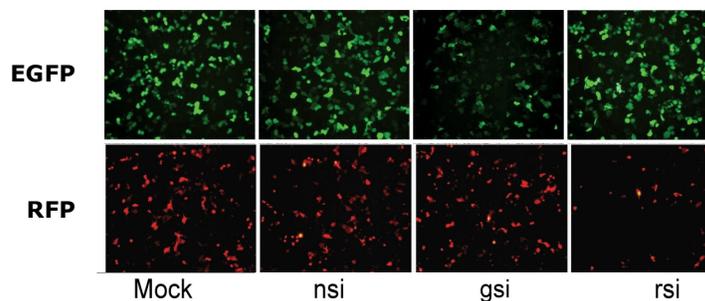
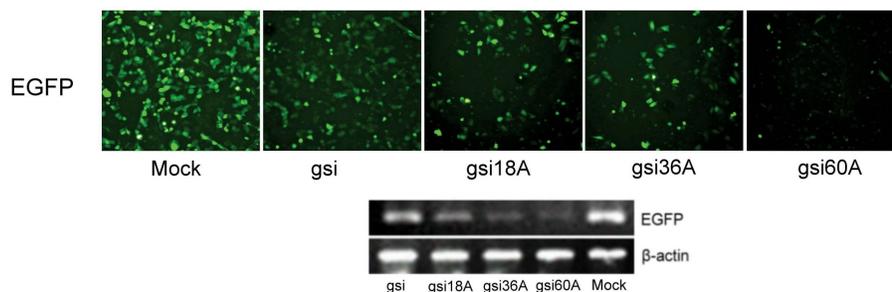
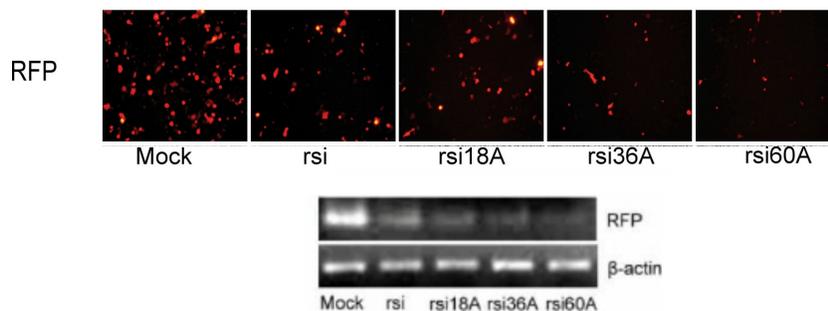
#### *The GAPDH primers (for qPCR):*

5'-GGGTGTGAACCATGAGAAGT-3' (forward)  
5'-GGCATGGACTGTGGTCATGA-3' (reverse)

## **RESULTS**

### **siRNAs with poly(A) tails enhance the silencing of exogenous reporter genes**

siRNA and siRNA-poly(A) were prepared with 18A, 36A or 60A tails on the 3' ends of their antisense strands for EGFP and RFP. These compounds were designated gsi, gsi18A, gsi36A, gsi60A, rsi, rsi18A, rsi36A, and rsi60A, respectively. The silencing efficiency of the prepared siRNAs on EGFP and RFP were firstly tested. In 24-well plates, MCF-7 cells were transfected with siRNA (40 nM) and reporter plasmid (200 ng). Under the fluorescence microscope, it was apparent that gsi could silence EGFP but had no effect on RFP, while rsi could silence RFP but had no effect on EGFP. Negative siRNA (nsi, a randomized non-specific control siRNA) had no effect on both EGFP and RFP (Figure 1A). Then, the silencing effect of gsi and gsi-poly(A) on EGFP were compared. Interestingly, the results showed that EGFP expression was silenced more efficiently by gsi-poly(A) than by gsi alone. Changes in EGFP mRNA levels displayed similar silencing effects (Figure 1B). The silencing activity of

**A****B****C**

**Figure 1.** Silencing effects of siRNA and siRNA-poly(A) against exogenous reporter genes. MCF-7 cells were co-transfected with EGFP or RFP plasmids (200 ng) and RNA (40 nM) in 24-well plates and visualized by fluorescence microscopy 48 hr later. RT-PCR analysis was done as described in Materials and Methods (Mock: no RNA treatment). **(A)** Fluorescence images of silencing effects of gsi, rsi and nsi on gene expression of EGFP or RFP respectively in MCF-7 cells. **(B)** Silencing effects of gsi and gsi-poly(A) on gene expression of EGFP in MCF-7 cells. Top: fluorescence images; Bottom: RT-PCR. **(C)** Silencing effects of rsi and rsi-poly(A) on gene expression of RFP in MCF-7 cells. Top: fluorescence images; Bottom: RT-PCR.

RFP siRNA and rsi-poly(A) on exogenous RFP expression was also investigated, and results indicated that RFP expression was inhibited by rsi and to a greater extent than by rsi-poly(A) (Figure 1C). Furthermore, the extent of inhibition appeared directly proportional to the length of the poly(A) tail.

#### Non-sequence-specific effects of siRNA-poly(A) on exogenous reporter genes

A specific silencing effect is an important characteristic of siRNA. To test the specificity of siRNA-poly(A), we co-

transfected MCF-7 cells with pEGFP-N2 and pdsRED2-N1 expression plasmids and gsi60A. The results showed that gsi60A inhibited the expression of both EGFP and RFP when compared with the MOCK group, but gsi inhibited EGFP expression only (Figure 2A). DNA was extracted from cells and assayed using semi-quantitative PCR for reporter gene plasmids. However, no obvious differences were discernable among the groups (data not shown). The inhibition by gsi60A was probably not a result of differences in transfection efficiency. While comparing gsi sense and antisense sequences with the RFP

mRNA sequence, we did not observe high homology or “seed regions” that are characteristic of microRNAs. The inhibition of RFP expression by *gsi60A* was apparently a non-specific effect. The strength of the inhibition was related directly to the length of poly(A) tail (Figure 2B). We obtained similar results with *rsi-poly(A)* and *nsi-poly(A)* (data not shown). The strength of the inhibition was related directly to the length of poly(A) tail. We suggest that the inhibition of exogenous EGFP and RFP expressions was non-specific.

#### Poly(A) tail causes non-specific inhibition

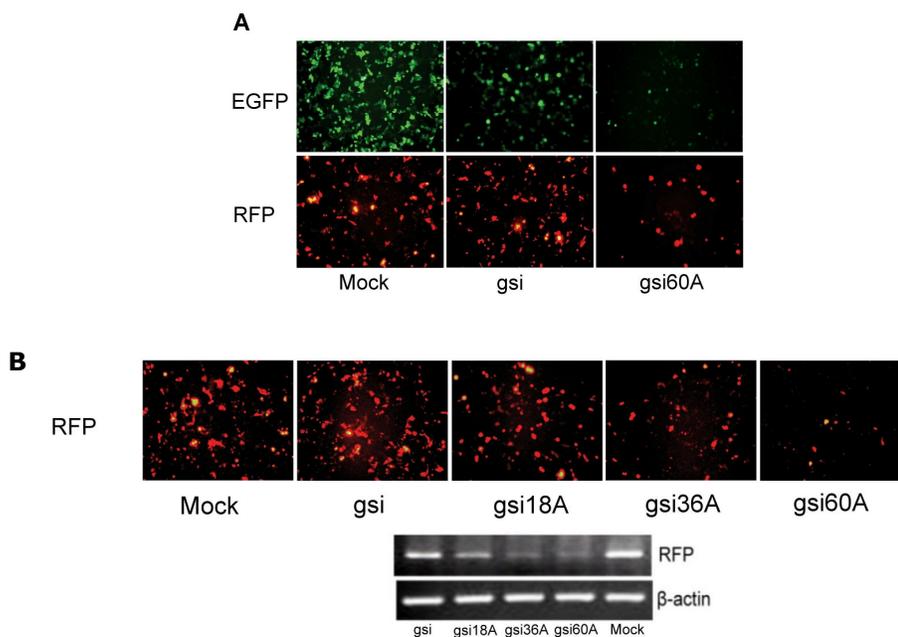
Since *gsi60A* exhibited improved silencing efficiency, the dependence of non-specific inhibition on poly(A) sequence was investigated. The polynucleotide chains 60U and 60N (N, randomized nucleotides) were substituted for 60A. We found that *gsi60U*, *gsi60N*, and *gsi* all inhibited EGFP expression but had no effect on RFP gene expression (Figure 3A). Interestingly, 60A itself conferred a non-specific silencing effect on exogenous EGFP gene expression; 60U, poly(G) and poly(C) showed no such effect (Figure 3B). We then used poly(A)s of various lengths (10A, 20A, 30A, 40A, 50A, and 60A) in a silencing activity assay. The results demonstrated that single strands of poly(A) that were longer than 50 nt could silence EGFP expression non-specifically (Figure 3C). We further compared the silencing effect of *gsi-poly(A)* and *nsi-poly(A)* on exogenous EGFP. The results showed that with the same length of poly(A) tails, *gsi-poly(A)* had stronger silencing activity than *nsi-poly(A)* (Figure 3D), which suggested that both of the EGFP specific-siRNA and the poly(A) contributed to the silencing effect of *gsi-poly(A)* on EGFP.

#### siRNA-poly(A) can activate double-stranded RNA-activated protein kinase (PKR)

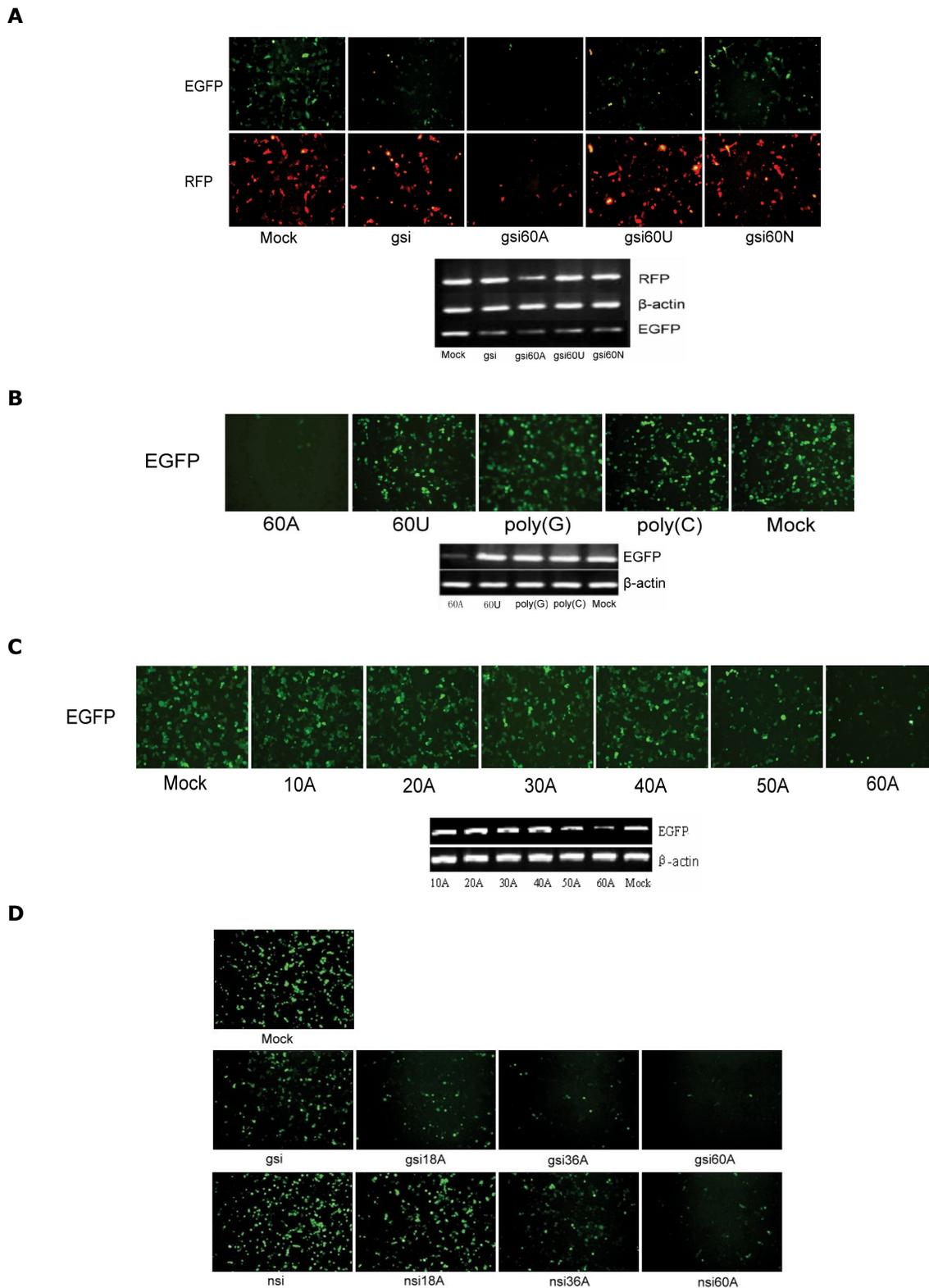
Interferon response is one of reasons for the nonspecific silencing effects of siRNA. Activation of PKR is the main event involved in this pathway. PKR is a double-stranded RNA-activated protein kinase. PKR uses its two N-terminal dsRNA binding domains as sensors to assist in inhibiting translation. It is well known that dsRNA strands that are longer than 30 bp can activate PKR. However, previous reports have demonstrated that short dsRNA, including 21 bp siRNA (Sledz et al, 2003; Bridge et al, 2003; Kim et al, 2004) and 16 bp dsRNA stems flanked by 10-15 nt single-stranded tails, could also activate PKR (Zheng and Bevilacqua, 2004). For these reasons, the effects of siRNA-poly(A) on PKR were investigated.

siRNA-poly(A)s activated PKR during an incubation with dephosphorylated PKR in the presence of [ $\gamma$ -<sup>32</sup>P]ATP, and the strength of activation of PKR was directly related to the length of poly(A) tail. However, siRNA or poly(A) of 60nt individually could not activate PKR *in vitro* (Figure 4, A and B). The activating ability of *gsi60A*, *nsi60A*, *gsi60U* and *gsi60N* on dephosphorylated PKR were also compared *in vitro*, and among these, only siRNAs with poly(A)s (*gsi60A* and *nsi60A*) could activate PKR (Figure 4C).

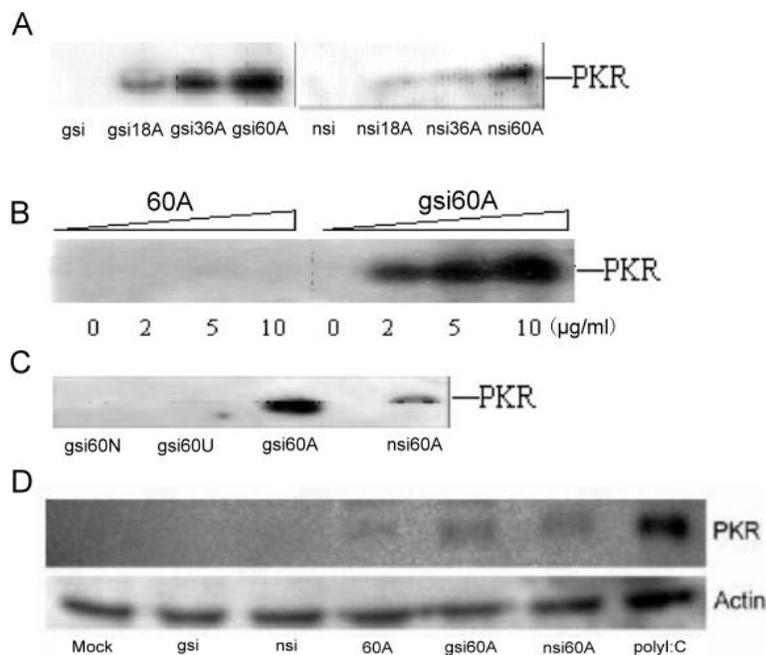
Activation of PKR was observed in MCF-7 cells following transfection with *gsi60A* and *nsi60A*, but the effects were weaker than those of polyI:C (polyinosinic acid-polycytidylic acid, a dsRNA analog). *gsi* and *nsi* did not activate PKR. Interestingly, 60A activated PKR weakly *ex vivo* (Figure 4D).



**Figure 2.** The nonspecific effect of siRNA-poly(A) on exogenous reporter genes. **(A)** Fluorescence images showing nonspecific inhibition of *gsi60A* on RFP expression. MCF-7 cells were co-transfected with EGFP plasmids (100 ng), RFP plasmids (100 ng) and RNA (40 nM) in 24-well plates and visualized by fluorescence microscopy 48h after transfection. (Mock: no RNA treatment). **(B)** Non-specific inhibition of *gsi* with different lengths of poly(A) on RFP gene expression. Top: fluorescence images; Bottom: RT-PCR. (Mock: no RNA treatment).



**Figure 3.** Poly(A) tail inhibits exogenous gene expression non-specifically. MCF-7 cells cultured in 24-well plates were co-transfected with RNA (40 nM) and EGFP plasmids (100 ng) or RFP plasmids (100 ng), respectively, and visualized using a fluorescence microscope 48h after transfection. RT-PCR analysis was done as described in materials and methods. (Mock: no RNA treatment). **(A)** Different effects of gsi60A, gsi60U and gsi60N on exogenous reporter gene expression. Top: fluorescence images; Bottom: RT-PCR analysis results. **(B)** Silencing effects of single-stranded poly(A), poly(U), poly(G) and poly(C) on EGFP gene expression. **(C)** Silencing effects of different lengths of single-stranded poly(A)s on EGFP gene expression. **(D)** Comparison of silencing effect of gsi-poly(A) with nsi-poly(A) on EGFP gene expression.



**Figure 4.** Activation of PKR by siRNA-poly(A) *in vitro* and *ex vivo*. **(A)** Effects of siRNA-poly(A) on activation of PKR *in vitro*. Dephosphorylated GST-PKR (0.5 µg) was incubated with 20 µM ATP, 10 µCi of [ $\gamma$ - $^{32}$ P]ATP and 10 µg/ml RNA in a total volume of 25 µl for 30 min at 23°C. After the reaction was complete, the samples were separated by 10% (w/v) SDS-PAGE and visualized by autoradiography. Lanes (left to right): gsi, gsi18A, gsi36A, gsi60A, nsi, nsi18A, nsi36A and nsi60A. **(B)** Different effects of gsi60A and 60A on activation of PKR *in vitro*. Lanes (left to right) corresponded to different concentrations of RNA: 60A (0, 2, 5 and 10 µg/ml) and gsi60A (0, 2, 5 and 10 µg/ml). **(C)** Different effects of gsi60A, gsi60N, gsi60U, and nsi60A on activation of PKR *in vitro*. **(D)** Effects of siRNA-poly(A) and poly(A) on activation of PKR *ex vivo*. MCF-7 cells were transfected with different RNAs. Cell lysates were prepared 48 hr after transfection, and western blot was done with primary antibodies specific against phosphopeptide Thr446.

#### Effects of siRNA-poly(A) on the stability of exogenous reporter gene mRNA

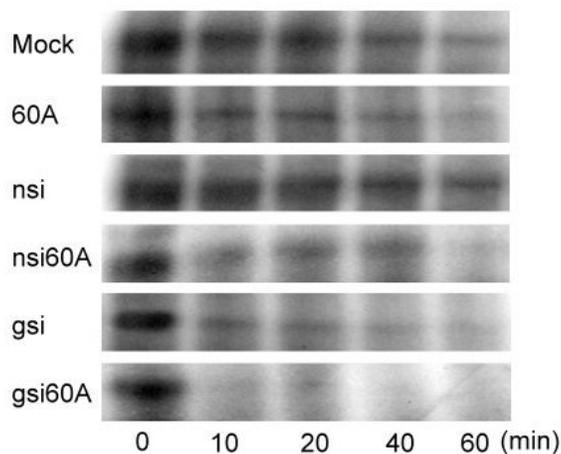
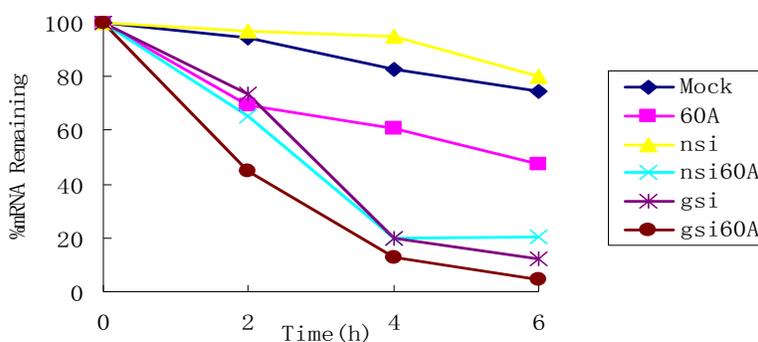
The effect of siRNA-poly(A) and poly60A on the stability of exogenous gene mRNA was investigated. First, mRNA decay was assessed by incubation of 5'- $^{32}$ P-labeled EGFP mRNA fragments with MCF-7 cell extracts and different RNAs *in vitro*. The results demonstrated an enhancement of mRNA degradation rates by nsi60A, gsi60A and poly60A, but not nsi. In other words, siRNA-poly(A), including poly60A, reduced exogenous gene mRNA stability in this cell-free decay system. gsi caused rapid decay of EGFP mRNA because of its RNAi effect (Figure 5A). Then *in vivo* mRNA decay assay was done with MCF-7 cells. After treatment, EGFP mRNA levels were analyzed by real-time RT-PCR at different times. The stability of EGFP mRNA in cells treated with gsi60A, nsi60A or 60A was lower than in cells treated with nsi. Gsi also silenced EGFP expression effectively because of its RNAi effect (Figure 5B).

#### DISCUSSION

In this study, it was demonstrated for the first time that single-stranded poly(A), following addition to the 3' end of an siRNA antisense strand could enhanced the silencing effect of the siRNA on exogenous reporter gene

expression. This enhancement was independent of the siRNA sequence and related directly to the length of the poly(A) strand. Our results showed that siRNA-poly(A) could activate PKR both *in vitro* and *ex vivo*. Although siRNA-poly(A) was a weaker activator of PKR than polyI:C, inhibition of translation by activated PKR could not be ruled out. Persengiev et al suggested that siRNAs affect nonspecific pathways that were overlapping but not identical to those regulated by interferon and polyI:C (Persengiev et al, 2004). That is, siRNAs do not trigger a true interferon response. In fact, this represented a possible mechanism of the non-specific enhanced RNA-silencing activity of siRNA-poly(A). Interestingly, single-stranded poly60A did not activate PKR *in vitro* but it could make PKR phosphorylation weakly *ex vivo*, representing a topic for further investigation. SiRNA-poly(A)s, including poly60A, also reduced the mRNA stability of the exogenous reporter EGFP gene, possibly representing another reason for their non-specific enhanced-silencing activity. Considering the feasibility of both explanations, the mechanism of action remains undefined.

Bernstein et al have suggested that the poly(A)-poly(A) binding protein complex influences mRNA stability *in vitro* (Bernstein et al, 1989). The transfected siRNA-poly(A) and poly60A might compete with poly(A) binding

**A****B**

**Figure 5.** Effects of siRNA-poly(A) on the stability of exogenous reporter gene mRNA. **(A)** Decay of EGFP  $^{32}\text{P}$ -mRNA *in vitro*.  $^{32}\text{P}$ -labeled EGFP mRNA fragments were incubated with 0.2  $A_{260}$  units of cellular extracts and 10 nM tiny RNAs in 20  $\mu\text{l}$  at 37°C. Autoradiographs were shown the decay of equal amounts of EGFP mRNA fragments following treatment with different RNAs. (Mock: no RNA treatment). **(B)** EGFP mRNA decay curves in MCF-7 cells following treatment with siRNA-poly60A and 60A. Total RNAs were isolated at different time points after treatment. The mRNA level of EGFP was determined by quantitative PCR and normalized with GAPDH. (Mock: no RNA treatment).

protein (PABP) to influence the stability of mRNAs of exogenous genes. In order to verify this, siRNA-poly(A)s targeting EGFP were co-transfected with GST-PABP1 plasmid (Lee and Bedford, 2002) in MCF-7 cells. We found that the silencing effect of siRNA-poly(A) on EGFP could not be reduced. RT-PCR results showed that exogenous PABP1 mRNA level was decreased to normal by siRNA-poly(A)s, but endogenous PABP1 mRNA level was not affected (data not shown). We also compared the effects of siRNA-poly(A)s on exogenous EGFP with stable expressed EGFP transgene. Surprisingly, the EGFP expression in stable transgene cells was not affected (data not shown). All this suggested that poly(A) tail had few effects on endogenous genes, but this needs to be further confirmed in the future.

Previously, Kawasaki and Taira added a poly(A) sequence to the 3' end of a hammerhead ribozyme, leading to extremely efficient cleavage of the target mRNA (Kawasaki and Taira, 2002). The poly(A) sequence of the

ribozyme interacted with eIF4A1, an RNA helicase, and cleaved the target mRNA regardless its secondary structure. Most recently, some interesting findings pointed to a relationship between poly(A) tails and the RNA interference pathway. When AGO2 or Dicer2 (essential factor for RNAi) in *Drosophila* was depleted, the reporter transgene EGFP protein levels rose and the mRNA poly(A) tails were shortened (Siomi et al, 2005). Humphreys et al reported that microRNAs controlled translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function (Humphreys et al, 2005). Wu et al found that microRNAs expedite poly(A) removal and lead to rapid mRNA decay (Wu et al, 2006). These results above suggested possible links between gene silencing and poly(A) tails beyond our traditional understandings.

We now know that many non-translatable mRNA-like RNA transcripts are present in cells (Erdmann et al, 2000). They are polyadenylated, spliced and lack long ORFs.

Many reports have suggested that these RNAs play a variety of structural, informational, catalytic and regulatory roles in the cell. However, only a few short mRNA-like RNAs (< 200bp) have been reported in human cells. As siRNA-poly(A) in this study looks like a short mRNA and non-specifically inhibits exogenous gene expression, we guessed the possibility that short mRNA-like RNA may have some important biological roles especially in mammalian cells.

In this paper, EGFP and RFP were used as exogenous reporter genes, but we believe that our results provide a functional connection between poly(A) sequence, siRNA and gene silencing. Our findings may be useful in siRNA designing especially when the targets are exogenous genes or against other invading virus RNAs.

## CONCLUSIONS

Single-stranded poly(A) tails on the 3' ends of siRNAs antisense strands have stronger silencing effects on exogenous genes than their corresponding parental forms. The enhanced silencing effect is related to the length of poly(A) but is non-specific. The nonspecific enhanced silencing effect may be related to the weak activation of PKR and the reduced stability of exogenous genes mRNAs. These results contribute the design of siRNAs and suggest the possible application of siRNA-poly(A) on cell defenses against exogenous genes or virus.

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## STATEMENT OF COMPETING INTERESTS

Ningsheng Shao has two pending patents on the methods of this paper.

## LIST OF ABBREVIATIONS

siRNA-poly(A): poly(A) tailed siRNA  
 gsi: EGFP siRNA  
 gsi18A: EGFP siRNA-18A  
 gsi36A: EGFP siRNA-36A  
 gsi60A: EGFP siRNA-60A  
 rsi: RFP siRNA  
 rsi18A: RFP siRNA-18A  
 rsi36A: RFP siRNA-36A  
 rsi60A: RFP siRNA-60A  
 nsi: Negative siRNA  
 nsi18A: Negative siRNA -18A  
 nsi36A: Negative control siRNA -36A

nsi60A: Negative control siRNA -60A  
 PKR: Double-stranded RNA-activated protein kinase  
 polyI:C : Polyinosinic acid:polycytidilic acid  
 PABP: Poly(A) binding protein

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