siRNA-mediated off-target gene silencing triggered by a 7 nt complementation

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ABSTRACT

A growing body of evidence suggests that siRNA could generate off-target effects through different mechanisms. However, the full impact of off-target gene regulation on phenotypic induction and accordingly on data interpretation in the context of large-scale siRNA library screen has not been reported. Here we report on off-target gene silencing effects observed in a large-scale knockdown experiment designed to identify novel regulators of the HIF-1 pathway. All of the three ‘top hits’ from our screen have been demonstrated to result from off-target gene silencing. Two of the three ‘siRNA hits’ were found to directly trigger down-regulation of hif-1α mRNA through a 7 nt motif, AGGCAGT, that is present in both the hif-1α mRNA and the siRNAs. Further analysis revealed that the generation of off-target gene silencing via this 7 nt motif depends on the characteristics of the target mRNA, including the sequence context surrounding the complementary region, the position of the complementary region in the mRNA and the copy number of the complementary region. Interestingly, the off-target siRNA against hif-1α was also shown to trigger mRNA degradation with high probability of other genes that possess multiple copies of the AGGCAGT motif in the 3′-untranslated region. Lessons learned from this study will be a valuable asset to aid in designing siRNAs with more stringent target selectivity and improving ‘hits-follow-up’ strategies for future large-scale knockdown experiments.

INTRODUCTION

siRNA is a powerful tool for studying the loss-of-function phenotypes in mammalian cells. The high potency of siRNA and its ease of use make it possible to carry out genome-wide gene silencing experiments to gain new insight in critical biological processes and to identify novel therapeutic targets (1–4). One of the perceived advantages of using siRNA as a functional genomics tool is its ability to silence genes in an sequence-specific manner. However, a growing body of evidence suggests that siRNA specificity is not absolute and off-target gene silencing can occur through different mechanisms, including global up/down-regulation of genes using high concentrations of siRNA (5,6), the induction of an interferon response (7), miRNA-like translational inhibition (8–10) and mRNA degradation mediated by partial sequence complementation (11). Some of the off-target effects can be eliminated using lower concentrations of siRNA, while others are preserved even when very low siRNA concentrations are employed (5,6,11). Because it is difficult to predict whether a particular siRNA will exhibit off-target effects, for practical applications of using siRNA for systematic knockdown experiment, one of the key questions is the full impact of the off-target gene regulation to phenotypic induction in the context of a large-scale siRNA library screen. Answers to this question will likely affect the interpretation of data from the primary screen and lead to different strategies for ‘hits-follow-up’.

HIF-1 is a heterodimeric transcription factor that consists of HIF-1β and HIF-1α subunits. The activation of HIF-1 under reduced oxygen pressure involves multiple steps including reduced degradation of HIF-1α, nuclear translocation and recruitment of cofactors such as p300/CBP. In some of these steps such as reduced degradation, oxygen appears to be the principle regulator (12–14). In other steps such as cofactor binding, both oxygen-dependent and independent mechanisms are involved. The direct involvement of HIF-1 in tumor development has been demonstrated by experimental manipulation of HIF-1 activity in tumor xenografts. These studies demonstrated that activation of HIF-1 promoted tumor growth, while inhibition of HIF-1 activity delayed tumor growth, suggesting that the HIF-1 pathway might be a promising area of intervention for cancer therapy (12–14).
Unfortunately, extensive studies on the oxygen-dependent regulatory pathway of HIF-1 activity thus far have failed to reveal any obvious targets that are deemed ‘druggable’.

In order to identify novel ‘druggable’ regulators of the HIF-1 pathway, we carried out a large-scale knockdown experiment using a synthetic siRNA library against 507 human kinases. Here we report on the top hits obtained in this screen. All the three ‘top hits’ are due to off-target gene silencing. Among these, two of the ‘siRNA hits’ were found to directly down-regulate the hif-1α mRNA through a 7 nt complementation.

MATERIALS AND METHODS

The siRNA library against 507 kinases

To obtain a list of reliable kinase sequences that are non-redundant, we used the list of protein kinase from the Incyte gene family and public domain sequences from the Protein Kinase Resource (PKR) at the San Diego Super-Computer Center (SDSC). A list of 799 kinase protein sequences was obtained from the Incyte. To derive the nucleic acid sequence of these proteins, the Incyte nucleic acid database was queried using the program TBLASTN. By comparing the sequences, redundant entries were removed, leaving 763 non-redundant kinase sequences. These sequences were compared to those in the Genbank reference sequence database (RefSeq). A total of 319 sequences had an exact match (>95% identity over the entire length of the sequence), 380 sequences were homologous and 64 sequences were classified as unique when compared to the sequences in the RefSeq database. From the public domain human kinase sequence, 663 sequences were obtained after removing multiple listings of the same sequence, potential polymorphisms and splice variants. Of these sequences, 250 were well characterized and were found in the RefSeq database. The final list of kinases was obtained by combining the Incyte (319) and public domain (250) sequences found in RefSeq with the unique Incyte sequences (64) to yield a total of 507 kinase sequences after the redundancies were removed.

The siRNAs against the kinase sequences were designed based on the rules suggested by Tuschl and co-workers (15). Sequences containing an AA ~75 nt from the start codon and with the G/C content between 40–70% were selected. In addition, siRNAs containing stem-loop structures predicted using Zucker’s RNA folding algorithm were eliminated. One thermodynamic rules similar to those implemented in the program TBLASTN. By comparing the sequences, redundant entries were removed, leaving 763 non-redundant kinase sequences. These sequences were compared to those in the Genbank reference sequence database (RefSeq). A total of 319 sequences had an exact match (>95% identity over the entire length of the sequence), 380 sequences were homologous and 64 sequences were classified as unique when compared to the sequences in the RefSeq database. From the public domain human kinase sequence, 663 sequences were obtained after removing multiple listings of the same sequence, potential polymorphisms and splice variants. Of these sequences, 250 were well characterized and were found in the RefSeq database. The final list of kinases was obtained by combining the Incyte (319) and public domain (250) sequences found in RefSeq with the unique Incyte sequences (64) to yield a total of 507 kinase sequences after the redundancies were removed.

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siRNA Library screening using the HIF-1 reporter assay

The HIF-1 reporter, pRL-FF, was constructed by inserting the hypoxia response element (HRE) from the enolase promoter into the pGL3-promoter vector (Promega, WI). For siRNA library screening, the HIF-1 reporter and a control reporter, pRL-TK (Promega, WI), were first transfected into H1299 cells grown in 10 cm plates using the Lipofectamine 2000 reagent (Invitrogen, CA). Three hours after transfection, the cells were trypsinized and plated into 96-well plate at 8,000 cells/well and incubated overnight to allow cells to attach and spread. siRNAs were transfected the following day at a final concentration of 100 nM (initial library screen) or 20 nM (in the subsequent retest experiment) using the Lipofectamine 2000 reagent. After transfection of siRNA for 24 h, the cells were transferred to an environment containing 2% O₂ and 5% CO₂ for an additional 15–18 h and then analyzed using the Dual-Glo luciferase assay system (Promega, WI). Using a siRNA against HIF-1α as a positive control and a siRNA irrelevant to the HIF-1 pathway as a negative control, a z’ factor of 0.58 was obtained, indicating that the HTS assay is very robust.

Human VEGF enzyme-linked immunosorbent assay

H1299 cells grown in 96-well plate were transfected with 20 nM of indicated siRNAs for 24 h and subjected to hypoxia treatment for an additional 15–18 h. Medium was collected and assayed for VEGF using a human VEGF-specific ELISA kit (R&D systems, MN).

Screen for potent shRNAs targeting GRK4, BTK and HK1

The cDNAs encoding full-length GRK4 (NM_182982), BTK (NM_000061) and HK1 (M75126) were cloned into the pcDNA3.1/V5-His vector (Invitrogen, CA) to generate expression vectors that produce V5-His tagged proteins. The shRNA expression constructs were generated using a protocol described previously (16). To select the most potent shRNA for each of the targets, the expression plasmid was co-transfected with an shRNA expression plasmid at a 1:1 ratio into H1299 cells. After post-transfection for 24 h the cells were harvested and analyzed by western blotting using the anti-V5 antibody. Synthetic siRNAs with identical sequences to the most potent shRNAs were obtained from Dharmacon and designated as new siRNAs [GRK4(N), HK1(N) and BTK(N)].

Western blotting

For western blotting, cells were directly lysed on 6-well plates in 1× Laemmli sample buffer (Bio-Rad). Proteins were separated by SDS–PAGE, transferred onto a PVDF membrane and western blotting was performed according to standard procedures using the mouse anti-HIF-1α mAbs (Pharmingen, CA), the monoclonal p53 antibody (Oncogene Science, NY) and the monoclonal β-actin antibody (Sigma, MO).

Quantitative RT–PCR (QPCR)

H1299 cells were transfected with 20 nM siRNA for 24 h and total RNA was extracted using the RNeasy mini kit (Qiagen, CA). QPCR analyses was performed using the ABI-7700 Sequence Detector instrument and the Platinum Quantitative RT–PCR ThermoScript One-Step System (Invitrogen, CA). The ABI’s ‘Assay on demand’ primer/probe mixtures were used for each of the following genes: hif-1α (HS00153153M1), GRK4 (HS00178384M1), RDX (HS00267954M1), Pias3 (HS00180666M1), Ppim1 (HS00260669M1), Disc1 (HS00218680M1), Ndufs1 (HS00192297M1), Abli (HS00245443M1), Rab6ip1 (HS00383858M1) and Nup210 (HS00227779M1). Each reaction was run in triplicates. The expression level for each sample was first normalized to the amount of GAPDH or β-actin and then to the experimental
control. The following primer/probe sets were used for the control genes. Human β-actin: β-actin_1316F (CTGGAAACGGTGAAGGTGACA), β-actin_1380R (CGGCCACATTGT-GAACCTTG) and β-actin_1337T ([FAM]CAGTCGGTTG-GAGGCAGACATCC(TAMRA)]. Human GAPD: hGAPD-3393F (AAATTCATGGCACCCTGC), hGAPD-3404R (GATGTTGATGGGATTTCCA) and hGAPD-3378T ([FAM]CAAGCTCCCTCGTCTAGCC(TAMRA)].

**RESULTS**

**Off-target gene silencing observed in a large-scale siRNA knockdown experiment**

In order to identify novel ‘druggable’ targets that regulate the HIF-1 signal pathway, we screened our siRNA library to identify genes that when knocked down, inhibit the HIF-1 pathway in a cell-based reporter gene assay. A Z’ factor of 0.58 was obtained using this assay, indicating that the assay is robust and well suited for HTS screening (data not shown). siRNA hits were defined as positive if they reduced luciferase activity to a value that had more than a 90% probability of being statistically different from that of the siRNA population in the testing plate. By screening a library of 507 siRNAs designed against the kinase family of enzymes using this reporter assay, we identified 83 siRNAs that down-regulated the HIF-1 reporter under hypoxic conditions. After eliminating siRNAs that behaved like general transcriptional inhibitors through a counter-screen using a constitutive reporter, pGL3-control, the remaining siRNA ‘hits’ were further characterized for their abilities to inhibit the production of the HIF-1 target, VEGF. Results from these studies revealed that siRNAs against GRK4, BTK and HK1 exhibited >40% inhibition of the HIF-1 reporter activity and the VEGF production under hypoxia without affecting the activity of the pGL3-control reporter, suggesting that these genes are involved in the hypoxia response mediated by HIF-1.

To ensure that the observed targets were related, several additional siRNAs against each of the targets were obtained and the correlation between the degree of target knockdown and the degree of inhibition of the HIF-1 reporter by each of the siRNAs was examined. Most of the additional siRNAs that we obtained failed to exhibit strong inhibition on the HIF-1 reporter activity. However, these siRNAs also caused less target knockdown compared to the original siRNAs in the kinase siRNA library (data not shown), suggesting that either a more complete knockdown of the target is required to affect the HIF-1 pathway or the phenotypes observed using the original siRNAs are due to an off-target effect. To distinguish between the two possibilities, we identified siRNAs that were able to knockdown the target to a higher degree than the original siRNA by screening a panel of shRNAs for their abilities to knockdown each target (data not shown). GRK4(N) and HK1(N), the siRNAs that are based on the most potent shRNA sequences against GRK4 and HK1, were found to be more efficient than the original siRNAs in knocking down these targets at both the endogenous mRNA level and the level of exogenously introduced epitope tagged proteins (Figure 1A and B, left and middle panels). However, none of these new siRNAs was able to inhibit the HIF-1 reporter activity under hypoxia (Figure 1A and B, right panels), demonstrating that the inhibition of the HIF-1 pathway by the original GRK4 and HK1 siRNAs was due to off-target effects. Similarly, the new BTK siRNA, BTK(N), knocked down a transiently expressed BTK protein to a higher degree than the original BTK siRNA, BTK(O) (Figure 1C, middle panel), but failed to inhibit the HIF-1 reporter activity under hypoxia (Figure 1C, right panel). In addition, QPCR analysis failed to detect any BTK mRNA in the cells that were used in the siRNA library screen (data not shown), which provided further evidence that the observed inhibition on the HIF-1 pathway by BTK(O) is an off-target effect. Taken together, all of the three ‘top hits’ from screening a library of 507 siRNAs were resulted from the off-target effects of siRNAs.

**A 7 nt complementation between the original GRK4(O)/BTK(O) siRNAs and the hif-1α mRNA triggers the degradation of hif-1α mRNA**

To gain more insight on how siRNAs generate off-target effects, we examined whether the original GRK4, BTK and HK1 siRNAs were able to regulate HIF-1α directly. Down-regulation of HIF-1α by the original GRK4 and BTK siRNAs was observed at both the protein and the mRNA levels, suggesting that these siRNAs might directly target the hif-1α mRNA (Figure 2A and B). Sequence analysis revealed the presence in the 3’-untranslated region (3’-UTR) of the hif-1α mRNA of two copies of a 7 nt motif complementary to 2–8 nt at the 5’ end of the antisense strands of the original GRK4 and BTK siRNAs (Figure 3A). This suggests that these two siRNAs might target hif-1α mRNA in these regions. To determine whether the 7 nt region is responsible for the off-target effect of the original GRK4 siRNA, an siRNA with 1 nt mutated in the 7 nt region was tested for its ability to knockdown HIF-1α [Figure 3A, GRK4(M)]. This mutated siRNA, GRK4(M), failed to decrease HIF-1α at the mRNA or protein levels or exhibit any inhibitory effects on the HIF-1 reporter activity (Figure 3B–D), indicating that the 7 nt region in the
original GRK4 siRNA was responsible for the observed off-target effect. Similar results were also obtained using BTK(M), a siRNA with a 1 nt mutation in the 7 nt motif of the original BTK siRNA (data not shown), suggesting that the same 7 nt motif is also critical for the off-target silencing of HIF-1α by the original BTK siRNA.

To distinguish whether the down-regulation of HIF-1α by GRK4(O) is due to direct targeting of the hif-1α mRNA or indirect targeting of an unknown HIF-1 regulator, we created a panel of luciferase reporters by inserting different forms of the hif-1α mRNA downstream of the coding sequence of luciferase [Figure 4, Luc, Luc-HIF-1α(ORF), Luc-HIF-1α(FL) and Luc-HIF-1α(3'UTR)]. A siRNA that targets the inserted hif-1α mRNA would be expected to degrade the luciferase mRNA produced from these constructs and lead to a reduction in luciferase activity. The original GRK4 siRNA was found to cause a reduction of luciferase activities when the reporters contain the 3'UTR of the hif-1α mRNA [Figure 5A, GRK4(O), Luc-HIF-1α(3'UTR) and Luc-HIF-1α(FL)]. In contrast, no reduction of luciferase activities was observed when the reporters only contain the coding region of the hif-1α mRNA [Figure 5A, GRK4(O), Luc-HIF-1α(ORF)]. As a control, a HIF-1α siRNA that targets the coding region of the hif-1α mRNA caused inhibition on the HIF-1 reporter activity when the reporters only contain the coding region of the hif-1α mRNA [Figure 5A, GRK4(O), Luc-HIF-1α(3'UTR)]. However, it is still possible that GRK4(O) might down-regulate the hif-1α mRNA indirectly through knocking down an unknown protein that interacts with the two 7 nt motifs and stabilizes the hif-1α mRNA in cells. To provide additional support of the hypothesis that the two 7 nt motifs in the hif-1α mRNA are responsible for GRK4(O)-induced gene silencing, we mutated the
HIF-1α(3’UTR) reporter to disrupt the two 7 nt box individually or in combination [Figure 5B, HIF-1α(3’UTR)m1, HIF-1α(3’UTR)m2 and HIF-1α(3’UTR)m3]. Mutations that disrupted both boxes were found to completely block the ability of GRK4(O) to inhibit the reporter [Figure 5B, GRK4(O), HIF-1α(3’UTR)m3], suggesting that the two boxes in the HIF-1α mRNA are the targeting sites of GRK4(O).

Compared to the reporter with both boxes disrupted, the reporters with either one of the regions mutated exhibited partial resistance to GRK4(O)-induced inhibition, indicating that the two boxes function independently in an additive manner [Figure 5B, GRK4(O), HIF-1α(3’UTR)m1 and HIF-1α(3’UTR)m2]. Similar results were also observed using the original BTK siRNA (data not shown), indicating that both BTK(O) and GRK4(O) target hif-1α mRNA at the same sites with similar mechanisms.

Because the two 7 nt boxes in the HIF-1α mRNA are in close vicinity, we examined whether the distance between the two boxes would affect the off-target gene silencing by
A panel of reporters were created by keeping the box2 unchanged while moving the box1 upstream or downstream of its original location [Figure 4, HIF-1α(3’UTR)Box1U200, HIF-1α(3’UTR)Box1D200 and HIF-1α(3’UTR)Box1U400]. It was found that moving the box1 upstream 200 or 400 bp did not affect the degree of silencing induced by GRK4(O) and moving the box1 downstream 200 or 400 bp only caused a slight reduction of silencing (Figure 5C). These results suggest that the space between the two 7 nt boxes is not a critical determinant of the silencing efficiency. We next examined the impact of the sequences surrounding the two boxes in the hif-1α mRNA on GRK4(O)-mediated silencing. A 50 bp fragment spanning the two 7 nt boxes from the hif-1α 3’-UTR was inserted into the Luc-HIF-1α(ORF)Box1/2 reporter [Figure 4, Luc-HIF-1α(ORF)Box1/2]. In this reporter, the distance from the stop codon of the luciferase coding sequence to the two 7 nt boxes was kept the same as in the HIF-1α(3’UTR) reporter. Surprisingly, GRK4(O) failed to exhibit any inhibitory effect on the Luc-HIF-1α(ORF)Box1/2 reporter (Figure 5D), suggesting that silencing by GRK4(O) is dependent on the sequences surrounding the box1 and box2 of the hif-1α mRNA.

Figure 4. Schematics of luciferase reporters contain different fragments/mutations of the hif-1α mRNA. The luciferase coding region is black colored. The hif-1α mRNA is gray colored. ‘TGA’ is the stop codon of the hif-1α mRNA (2763–2765 nt). The numbers are based on the hif-1α mRNA entry NM_001530. The black boxes represent the two 7 nt boxes, Hif1Box1 and Hif1Box2. Luc-Hif-1α (ORF), Luc-Hif-1α (FL), Luc-Hif-1α (3’UTR) contain the open reading frame of the hif-1α mRNA, the full-length hif-1α mRNA (from the start ATG to the end) and the 3’-UTR of the hif-1α mRNA, respectively. Luc-Hif-1α (3’UTR)m1, Luc-Hif-1α (3’UTR)m2 and Luc-Hif-1α (3’UTR)m3 contain mutations that disrupt the first, second or both 7 nt boxes in the 3’-UTR of the hif-1α mRNA. In Luc-Hif-1α (3’UTR)Box1U200, Luc-Hif-1α (3’UTR)Box1U400, Luc-Hif-1α (3’UTR)Box1D200 and Luc-Hif-1α (3’UTR)Box1D400, the first 7 nt box, HifBox1, was moved upstream 200–400 bp, or downstream 200–400 bp from its original position. In the Luc-Hif-1α(ORF)Box1/2 reporter, a 50 bp fragment spanning the two 7 nt boxes in the hif-1α 3’-UTR was inserted into the hif-1α open reading frame. The distance from the stop codon of the luciferase coding sequence to the two 7 nt boxes was kept the same in the Luc-Hif-1α(ORF)Box1/2 reporter as in the Luc-Hif-1α (3’UTR) reporter.
Off-target silencing of multiple endogenous genes by the GRK4(O) siRNA

Because gene silencing mediated by the 7 nt boxes in GRK4(O) appeared to be dependent on the sequence context in the target, we next examined whether GRK4(O) could silence genes other than HIF-1α that also possess multiple copies of the 7 nt motif, AGGCAGT. A BLAST analysis using AGGCAGT against human RefSeq identified 726 genes with more than one copy of the 7 nt motif in the coding region and 486 genes with more than one copy of this motif in the 3′-UTR. Eight genes with multiple copies of the AGGCAGT sequence in the coding region or 3′-UTR were selected for qPCR analysis of gene knockdown by GRK4(O). Two out of the four genes with the AGGCAGT motif in 3′-UTR exhibited more than a 40% reduction of mRNA upon the transfection of GRK4(O) but not GRK4(N) (Figure 6, PPM1F and DISC1). One of the four genes with the 7 nt motif in 3′-UTR was down-regulated by both GRK4(O) and GRK4(N) (Figure 6, RDX), suggesting that this gene might happen to be a GRK4 regulated gene. In contrast, none of the four genes with the targeting sites in the coding region were inhibited by GRK4(O) (Figure 6, RAB6IP1 and NUP210). These results suggest that genes with multiple partial complementary sites in the 3′-UTR to 2–8 nt of the antisense strand of a siRNA have a good probability of being silenced. However, not every gene with similar complementary sites in 3′-UTR will be silenced, as demonstrated by the lack of PIAS3 knockdown by GRK4(O) (Table 1, PIAS3).

DISCUSSION

Although siRNA-mediated off-target effects have been reported, the extent of the off-target effect is generally believed to be relatively small. Therefore, it was surprising to find that all of the ‘top hits’ from our screen were due to off-target effects of the siRNAs. A probable explanation is that the screening process enriches siRNAs that produce a particular phenotype. Owing to the large number of siRNAs in the library, the small off-target effects of the siRNAs are amplified, leading to a large percentage of off-target ‘hits’ in the screening. Reducing the siRNA concentration in the screen is insufficient to alleviate the off-target effects. In our hands, all of the off-target ‘hits’ inhibited the HIF-1 pathway at a concentration as low as 5 nM. Therefore, verifying the hits with multiple siRNAs and using non-RNAi-based methods such as rescuing the phenotype with a siRNA resistant mutant of the target are absolutely critical. In addition, due to the large percentage of the off-target hits generated in the screening, using a redundant library without pooling in the primary screen could significantly reduce the efforts required to eliminate off-target false positives and therefore, will be a more efficient design than using a pooled library.

It is intriguing that a 7 nt complementation between the siRNA and the target is sufficient to cause gene silencing. Although the silencing effect is relatively weak with the presence of only one partially complementary site, the presence of multiple sites appears to amplify the silencing effect. Another finding from our study is that sequences surrounding the siRNA targeting sites are also important for the silencing effect.
The position of sequence complementation between GRK4(O)/BTK(O) or the MAPK14 siRNAs and their unintended targets makes it more difficult to predict the off-target effect of a given siRNA.

The possibility for siRNAs to trigger gene silencing with very limited complementation has been suggested in the expression profiling study carried out by Jackson et al. (11), in which, the expression of a small set of genes was down-regulated with similar kinetics to MAPK14 and several of these potentially off-target regulated genes were found to have sequence identity to the MAPK14 siRNA only at 12–18 nt of the sense strand (equivalent to 2–8 nt of the antisense strand). Our results are consistent with Jackson et al. in which we have shown that a 7 nt complementation between a siRNA and a target is necessary and in many cases sufficient to cause mRNA degradation of the target.

The position of sequence complementation between GRK4(O)/BTK(O) or the MAPK14 siRNAs and their unintended targets remarkably resembles the sequence complementation between miRNAs and their targets. The 5' ends of many miRNAs have been shown to contain a 7 nt sequence that are complementary to several classes of sequence motifs known to mediate negative post-transcriptional regulation (19–21). In addition, one of the common features of the GRK4(O)/BTK(O) siRNAs and the MAPK14 siRNA used in Jackson’s study is the high G/C content at positions 2–8 of the antisense strand, suggesting a high thermodynamic stability of this region. This is also consistent with a recent report that miRNAs with high thermodynamic stability at the 5' region are sufficient to inhibit the translation of their targets without any extensive complementation at the 3' region (22). The ability of siRNAs to enter the miRNA pathway to inhibit a target on the translation level has been reported (9,10). Interestingly, in this study the GRK4(O)/BTK(O) siRNAs triggers off-target mRNA down-regulation instead of translation inhibition. It was recently reported that some miRNAs down-regulate large numbers of target mRNAs, which indicates that miRNAs can also regulate their biological targets at the mRNA level (23). In addition, Jing et al. recently reported that miRNA together with the RISC complex can associate with proteins that are involved in modulating mRNA stability. Therefore, it is conceivable that the off-target siRNA could behave like a miRNA to interact with its undesired target through the 7 nt motif, which may result in the recruitment of the RISC complex to the target mRNA and subsequently the recruitment of proteins that are involved in regulating mRNA stability (24). Taken together, we speculate that the GRK4(O)/BTK(O) siRNAs might adopt the same mode of action as a miRNA to interact with and down-regulate the mRNA of its unintended target, HIF-1α. Considering the importance of thermodynamic stability in the 5' region of a miRNA for target recognition, it might be possible to improve the selectivity of siRNA by choosing siRNAs with lower thermodynamic stability at the 5' region of the antisense strand. On the other hand, since a partial complementation between a siRNA and the 3'-UTR of a gene appeared to trigger off-target silencing with high probability, designing siRNAs with minimal complementation to the 3'-UTR of all the genes in the genome will also likely improve the specificity of siRNA.

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