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Research article

A STUDY ON THE FUNDAMENTAL FACTORS DETERMINING THE EFFICACY OF siRNAs WITH HIGH C/G CONTENTS

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Abstract: Although there are many reports about the efficacy of siRNAs, it is not clear whether those siRNAs with high C/G contents can be used to silence their target mRNAs efficiently. In this study, we investigated the structure and function of a group of siRNAs with high C/G contents. The results showed that single siRNAs against the Calpain, Otoferlin and Her2 mRNAs could induce different silencing effects on their targets, suggesting that the accessibility to target sequences influences the efficacy of siRNA. Unexpectedly, a single siRNA could target its cognate sequence in the 3'UTR of EEF1D or the 5'UTR of hTRF2 or CDC6. Their interaction induced different modes of gene silencing. Furthermore, the introduction of mutations into the 3' end of the passenger strand showed that the position and number of mutated nucleotides could exert some influence on the efficacy of siRNA. However, these mutations did not completely block the passenger strand from exerting its RNAi effect. Interestingly, our findings also indicated that the target mRNA might play

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Abbreviations used: Ago – argonaute; C – cytosine; Calp – calpain; ds – double-strand; G – guanine; Her2 – v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; HRP – peroxidase; Otof – otoferin; RISC – RNA-induced silencing complex; RNAi – RNA interference; RNase – ribonuclease; siRNA – short interfering RNA; TMB – 3,3',5,5' tetramethylbenzidine

essential roles in maintaining or discarding the guide strand in RISCs. Thus, the conclusion could be drawn that favorable siRNA sequences, accessible target structures and the fast cleavage mode are necessary and sufficient prerequisites for efficient RNAi.

Key words: siRNA, RNAi, mRNA, Local structure, Gene silencing

INTRODUCTION

RNA interference (RNAi) is a double-stranded (ds) RNA-dependent process involved in post-transcriptional gene silencing [1]. During the initial step of the process, a ribonuclease (Rnase) III-like enzyme called Dicer cleaves long dsRNA into short interfering RNA (siRNA) of 21~31 nt in length [2-4]. With the assistance of R2B2, the resulting siRNA duplexes generated within cells or synthesized *in vitro* are assembled into the RNA-induced silencing complex (RISC). Elbashir *et al.* [5] demonstrated that siRNAs introduced into cells could effectively induce mRNA degradation without the interferon response. With the discovery of the crystallographic structure of the Ago protein [6, 7], a core protein of RISC, it has become clear that protein-protein interaction is not part of the functioning of the RISC. The RISC is where the Paz domain recognizes and binds to the 2-bp 3' overhang of siRNAs, and the PIWI domain mediates both the interactions with the 5' end of siRNA and the catalytic activity responsible for the cleavage of target mRNA during RNA interference [8-11]. It is now well known that RNAi is a powerful tool for identifying gene function and developing potential therapeutic agents [12, 13]. Therefore, the design of highly effective siRNA is very important in meeting the aims of basic research and clinical application.

The secondary structure of the mRNA target and the sequence composition of siRNA likely contribute to the efficacy of siRNAs in gene silencing. The relationship between the siRNA context and its efficacy at inducing the degradation of RNA targets in human and mouse embryonic stem cells and in *Drosophila* cells has been examined [14]. Previous studies indicated that a low G/C content within the target regions of the mRNAs was closely associated with efficient gene suppression directed by siRNAs [15]. Most of the highly effective siRNAs were found to fall within the G/C content ranging from 36 to 52% [16]. Some observations demonstrated that the low internal stability of the sense 3' end facilitated strand selection and target degradation [17]. Reynolds *et al.* [18] and Jagla *et al.* [19] reported that a low G/C content might avoid areas of highly stable secondary structure, and that special localization of AT in siRNA could predict the effects of gene degradation even though there was no consideration of correlation between the secondary structure and RNAi.

The constraint of the secondary structure of the target mRNA has been shown to influence the activity of both ASOs and siRNAs [20-22]. Recently, some approaches have yielded results that further demonstrate the importance of the target structure and its accessibility for the function of the siRNAs [23, 24].

Several lines of investigation have provided evidence that the secondary structure can indeed have a negative influence on RNAi [25, 26]. On the other hand, computational predictions have been developed to address this problem according to the principle of free energy minimization. The established algorithms can compute the optimal folding and possible suboptimal folding [22, 27]. Recently, Ding *et al.* [28] developed an algorithm based on a precise statistical sampling of the folded RNA, which provides computational tools for target accessibility prediction and for the rational design of siRNAs. Heale *et al.* [24] reported that a novel approach for the determination of mRNA secondary structures could account for 80% of the non-functional siRNA target sites according to the combination of predicted strong secondary structures with duplex-end energies.

It is well known that the efficacy of siRNA-directed gene silencing varies markedly depending on the siRNAs and the different regions of the mRNA they are targeting [19, 25, 29]. The factors determining the siRNA-mediated silencing of the mRNA target have not been fully elucidated [30], although a number of studies demonstrated that sequence characteristics, the free energy of the siRNA duplex, and the secondary structure of the target mRNA influence the efficacy of the siRNA. To clarify how many fundamental factors determine siRNA efficacy and what the underlying mechanism is, we attempted to use siRNAs with high C/G contents to explore the influences of strand preference, secondary structure choice, complementary degree and silencing mode selection.

MATERIALS AND METHODS

Cell culture and transfection

Human breast cancer cell lines SK-BR-3 and MCF-7 were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM (Life Technologies) supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 12% FBS at 37ºC in a humidified atmosphere containing 5% CO_2. The day before transfection, the cells were trypsinized, diluted with fresh medium without antibiotics, and seeded into 6-well culture plates (approximately 10^6 cells/well). Sense and antisense strands of 21-nt siRNAs were preferably synthesized by Shanghai GenePharma (Shanghai, China). The transfection of synthetic siRNA duplexes was carried out using lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Different doses of siRNA duplexes against the target mRNAs were applied according to the experimental requirements, with mock siRNA taken as a control. 24 to 48 h after transfection, a cell lysate was prepared. To silence the expression of the endogenous genes, each synthetic siRNA duplex against the target genes was transfected into the cells. The sequence of mock siRNA was 5'-AAUAGUGUAUACGGCAUGCTT-3'.

Reverse transcription-PCR and real-time quantitative PCR

The expression of the relevant genes was done via the RT-PCR method [13]. Total RNA was isolated with TRI REAGENT (Molecular Research Center) according to the manufacturer's instructions. 2 μg of total RNA was then reverse transcribed into single-stranded DNA with M-MLV reverse transcriptase (Promega). The resulting cDNAs were subjected to PCR amplification in 20 μl mixtures with specific primers (1 μM each) for each gene. The primers used are listed in Tab. 1. Beta-actin was used as an internal control. Generally, 35 amplification cycles (94ºC for 45 sec, 55ºC for 30 sec and 72ºC for 30 sec) were performed using Promega *Taq* DNA polymerase. The PCR products were separated by 1.5% agarose gel electrophoresis. Cycle numbers were optimized in several experiments with the determination of the linear phase PCR reaction. All the experiments were duplicated and repeated at least three times. The band density was quantitated using Alphalmager 2200 analysis software.

The real-time quantitative PCR (BioRad iCycler MyiQ Real Time thermocycler) was performed as described in the manufacturer's instructions, using SYBR Green PCR Master Mix (Applied Biosystems). Cycling conditions included an initial denaturation at 94ºC for 5 min, followed by 50 cycles of 94ºC for 30 sec, 62ºC for 30 sec, and 72ºC for 25 sec, with a final extension of 72ºC for 10 min.

Tab. 1. The sequences of the primers used in RT-PCR.

Gene	Forward primer	Reverse primer
β -actin	5'-AGCCCTGGCTGCCTCAAC-3'	5'-TGGCGCTTTTGACTCAGGAT-3'
Her2a	5'-AGCCGCAGTGAGCACCAT-3'	5'-GGCATTGGTGGGCAGGTA-3'
Her2b	5'-CCCTCTGACGTCCATCATCT-3'	5'-CAAGCACCTTCACCTTCCTC-3'
Calpain	5'-GCCATTGGCTTTGACATCTA-3'	5'-GAA GGTTGTGGGGATGATGA-3'
Otoferlin	5'-GTTGGGGCTGCTCATGTT-3'	5'-CAGGGGAGATGGGAA AGAGT-3'
CDC6	5'-CCCGTTTGACAAAGGTGTTT-3'	5'-ACCTTAATGGCTGAGCATGG-3'
HTRF2	5'-AGAAGAAAGCGAGTGGGTC-3	5'-TATTTCTGGCACTGCACAAGC-3'
EEF1D	5'-ACGTGTTAGCCAGGATGGTC-3'	5'-TCTCTGCCTGGTCGAAAAGT-3'

Tab. 2. The sequences of the primers used for quantitative PCR.

Name	Sequence
Her-2 construct1 forward	5'-GCTCCGCCACCTCTACCA-3'
Her-2 construct1 reverse	5'-GTTGTGAGCGATGAGCACGTA -3'
Calpain-5 construct forward	5'- GATGAAGTCCTGATCTGCATCCA-3'
Calpain-5 construct reverse	5'-CCTTGTGCTGCAGGCTGTG-3'
Otoferlin construct forward	5'-CTCATCTGCACCCGGTACAAG-3'
Otoferlin construct reverse	5'-AAGAGTCCAAGCCACTGAAAGG-3'
Control construct forward	5'-GGGCTGCTTTTAACTCTGGTAAAG-3'
Control construct reverse	5'-CCATGGGTGGAATCATATTGG-3'

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used are listed in Tab. 2. To perform the analysis of the relative expression of these three genes using real-time PCR, we adopted the relative quantitative method by normalizing to GAPDH expression levels. Then the percentages of these three mRNAs in siRNA-transfected cells relative to those in mock-transfected cells were calculated. All of the experiments were performed in triplicate and the standard deviation was calculated.

RNA secondary structure prediction

For sequence searches and analysis, we used NCBI resources (http://www.ncbi.nlm.nih.gov). To predict the secondary structures of the target sequences, we employed the mfold program version 3.2. This program was designed to determine the optimal and suboptimal secondary structures of RNA, and to count free energy contributions for various secondary structure motifs. Generally, the local regions of the target mRNAs that have a target sequence in the center and are 120 nt or 500 nt in the length were used for the folding analysis. Those secondary structures of the target region with the free energy minimum and a similar conformation in twice folding were considered a stable structure, and selected for further experimental analysis. Otherwise, they were discarded.

Luciferase assays

pRL-TK vectors (Promega) with or without the target sequence were cotransfected with mock siRNA or S-siRNA. Cells were harvested and lysed 24 h after transfection, and *Renilla* luciferase activities were measured consecutively using the dual luciferase assays (Promega), according to the manufacturer's instructions using a Berthold Lumat 4097 luminometer (Perkin Elmer Life Sciences, Carlsbad, CA).

Western blotting

Cells were washed twice with cold PBS and then extracted in 20 μl of RIPA lysis buffer (50 mM Tris-HCl pH 7.5; 1% NP-40; 150 mM NaCl; 1 mg/ml aprotinin; 1 mg/ml leupeptin; 1 mM Na_3VO_4; 1 mM NaF; 1 mM PMSF) at 4ºC for 30 min. Total protein was resolved on 10% SDS-polyacrylamide gel eletrophoresis and bands of protein transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham). The membrane was blocked with 5% nonfat milk TBS buffer overnight at RT, and incubated for 2 h with primary antibodies. The expression of beta-actin was used as a loading control. The antibodies used included beta-actin (Santa Cruz Biotechnology), Her2 (Neomarkers), and Otoferlin (Orbigen). The membranes were then incubated for 1 h with HRP-conjugated rabbit anti-goat secondary antibody. Immunocomplexes were visualized with an ECL kit.

ELISA

To quantify the Her2 and Otoferlin protein in the experimental and control cases, ELISA was done using affinity-purified anti-Herb2 and Otoferlin Ab. Cells (6×10^6) were extracted in 600 µl RIPA buffer after 48 h of culture with siRNA. The starting volume was concentrated to 30 µl with Nanosep centrifugal devices (Pall life science) and diluted to 150 µl in CBS buffer. Different samples from experimental and control cases were added to a pretreated ELISA plate, and incubated at 37ºC for 2 hours. Herb2 or Otoferlin proteins were detected in each sample by incubating biotinylated anti-Herb2 or Otoferlin Ab and peroxidase (HRP)-labeled avidin (Santa Cruz Biotechnology, Inc) with 3,3',5,5' tetramethylbenzidine (TMB) solution. The optical density at 450 nm (OD_{450}) was measured with Multiskan MK3 (Thermo Labsystems).

RESULTS

An outline of the siRNAs employed in the study

To identify whether the specific types of siRNA duplex, the secondary structures of target mRNA and/or other factors play critical roles in siRNA-mediated gene silencing, we designed and chemically synthesized four groups of siRNAs. In group 1, both the sense and antisense strands of these siRNAs could target their respetive cognate genes. For example, the sense strand of H1N-siR can form 19 nt base-pairings with Calp-mRNA and 15 nt pairs with Otof-mRNA, while the antisense strand of this siRNA makes a perfect match with Her2-mRNA (Column 3 in Tab. 3). For the exposure extent of a local segment of an mRNA, we employed hairpin, stem, poor or good exposures for the different secondary structures of the targets (Column 4 in Tab. 3). To investigate whether the introduction of base mutations into the 3' end of the sense strand can enhance the efficacy of siRNAs, we constructed three different groups of siRNAs that are composed of wild and mutant types. It has been shown that higher CG pairs at one end of the siRNA duplex can increase the stability of that end. Our results (Column 5 in Tab. 3) were consistent with this observation. In order to better evaluate other factors affecting the efficacy of siRNA, the siRNA duplexes with a higher C/G content (> 53%) were employed (Column 6 in Tab. 3). In the last column, we gave the silencing efficacy of the various siRNAs used in this study according to Real Time-PCR and Western blot analysis.

As shown in Fig. 1, the RT-PCR assay showed that the target mRNA levels were down-regulated to different extents in SK-BR-3 cells transfected with the corresponding siRNAs for 24 hours, while mock siRNA was ineffective in the mRNAs, confirming that these siRNAs can specifically induce target mRNA silencing. Quantitative analysis (Fig. 1B) also indicated that transfection of the siRNAs targeting Her2, Otoferlin and Calpain mRNAs resulted in a decrease in Her2, Otoferlin and Calpain levels in a dose-dependent manner. Thus, to suppress target mRNAs effectively and decrease the toxicity of siRNAs, we used 100 ng of siRNAs for subsequent experiments unless a special amount was indicated.

Tab. 3. The features of the siRNAs used in this study.

siRNA name	Sequence	Target mRNA (base-pairing No.)	RNA structure	CG pairs in 5' or 3' ends	% of GC	Efficacy
Group-1						
H1N-siR	5'GGAGCUGGCGGCCUUGUGCTT	Calpain (19nt) Otoferlin (15nt)	Poor exp. Good exp.	4/3	74%	26% 30%
	TTCCUCGACCGCCGGAACACG	Her2 (19nt)	Stem			50%
H1M1-siR	5'GGAGCUGGCGGCCUAAUGCTT	Calpain (17nt) Otoferlin (16nt)	Poor exp. Good exp.	4/2	71%	20% 35%
	TTCCUCGACCGCCGGAACACG	Her2 (19nt)	Stem			55%
H1M2-siR	5'GGAGCUGGCGGCCUUAUGCTT	Calpain (18nt) Otoferlin (16nt)	Poor exp. Good exp.	4/2	71%	23% 37%
	TTCCUCGACCGCCGGAACACG	Her2 (19nt)	Stem			53%
H1M3-siR	5'GGAGCUGGCGGCCUUCAGCTT	Calpain (17nt) Otoferlin (17nt)	Poor exp. Good exp.	4/2	74%	15% 50%
	TTCCUCGACCGCCGGAACACG	Her2 (19nt)	Stem			68%
H1M5-siR	5'GGAGCUGGCGGCCUUCAUGTT	Calpain (15nt) Otoferlin (19nt)	Poor exp. Good exp.	4/0	71%	13% 85%
	TTCCUCGACCGCCGGAACACG	Her2 (19nt)	Stem			73%
H1M5-siR	5'GGAGCUGGCGGCCUUGUGCTT	Calpain (19nt) Otoferlin (15nt)	Poor exp. Good exp.	2/3	68%	40% 83%
	TTAAUCGACCGCCGGAACACG	Her2 (17)	Stem			53%
Group-2						
H2N-siR	5'UCUCUGCGGUGGUUGGCAUTT TTAGAGACGCCACCAACCGUA	Her2(19nt)	Stem	3/3	58%	55%
H2M-siR	5'UCUCUGCGGUGGUUGAGAUTT TTAGAGACGCCACCAACCGUA	Her2(19nt)	Stem	3/1	53%	70%
Group-3						
H3N-siR	5'CCGCUGACACCUAGCGGAGTT TTGGCGACUGUGGAUCGCCUC	Her2(19nt)	Hairpin	5/5	68%	30%
H3M-siR	5'CCGCUGACACCUAGCAUAGTT TTGGCGACUGUGGAUCGCCUC	Her2(19nt)	Hairpin	5/3	63%	41%
Group-4						
S-siR	5'GCCUCCCAAAGUGCUGGGATT		Hairpin	5/4	67%	45%
	TTCGGAGGGUUUCACGACCCT	CDC6(19nt) EEF1D(19nt)	Hairpin			68%

The mutated nucleotide in the sequence column is underlined. The number of C/G pairs was calculated by enumerating the C/G pairs out of 6 terminal pairs at either end of the duplex.

Either strand of an siRNA can be functional

To investigate whether the two strands of an siRNA duplex with high C/G contents can be unwound to directly degrade endogenous targets in cells, we designed a unique H1N-siR, and assayed the silencing ability of the sense

and antisense strands of the siRNA duplex against different endogenous mRNAs (Tab. 3). In this siRNA duplex, the antisense strand directs Her2 mRNA cleavage, while the sense strand targets Calpain and Oterfolin mRNA (Tab. 3 and Fig. 1A). The same is true for the other siRNAs in group 1, including H1M1-siR, H1M2-siR, H1M3-siR, H1M4-siR and H1M5-siR. The antisense strand of H1N-siR is completely complementary to the coding

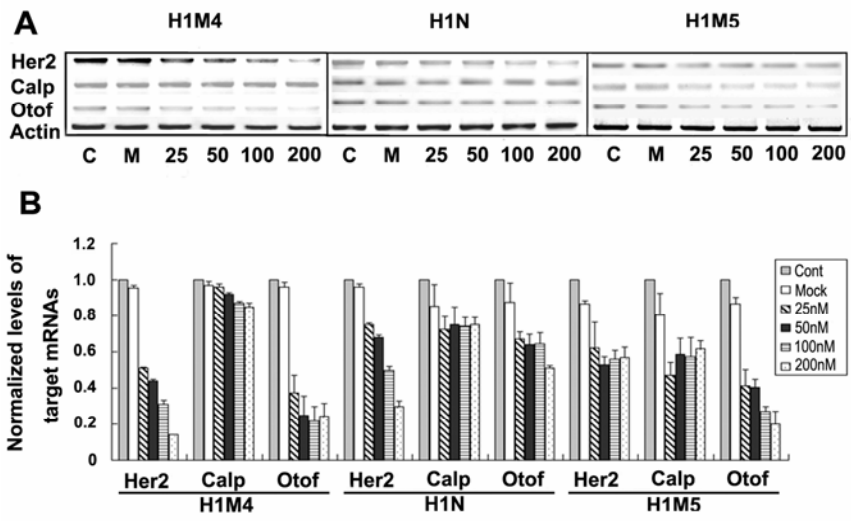

Fig. 1. A single siRNA duplex can inhibit three endogenous mRNAs in a dose-dependent manner. A – RT-PCR analysis of SK-BR-3 cells transfected with or without siRNA duplexes including wild type H2N and mutated H2M4 and H2M5. When SK-BR-3 cells were at 70-80% confluence, cells were transfected with different doses of siRNAs at 25, 50, 100, and 200 ng. Meanwhile, 200 ng mock siRNA (M) was introduced into the cells as a control (C). B – Based on the results of the RT-PCR, a quantitative analysis was carried out to determine the extent of the target mRNA reduction induced by siRNAs. The mean value is reported from three independent transfection experiments. Error bars show variations among the experiments as the standard deviation of the mean. The extent of target mRNA reduction was normalized by the percentage of the transcript level in siRNA-transfected cells relative to the transcript level in control cells.

sequence of the Her2 gene, and induced a decrease in both the Her2 gene and protein levels (Figs 1 and 2). Interestingly, the sense strand of the H1N-siRduplex was also a functional strand in silencing the endogenous gene. As shown in Figs 1 and 2, H1N-siR with partial complementarity to the target region of Otoferlin slightly inhibited the expression of Otoferlin, while H1M4-siR with full base matches suppressed the expression of Otoferlin mRNA by about 85% (Tab. 3). Control experiments showed that mock siRNA duplexes did not alter the Otoferlin mRNA and protein levels (Figs 1 and 2). Moreover, H2N-siR-siR, H2M-siR, H3N-siR and H3M-siR were completely ineffective in

suppressing the expression of Otoferlin mRNA, suggesting that the sense strands of H1N-siR and its mutants could specifically inhibit the activity of Otoferlin mRNA. This finding provided clear-cut evidence that the two strands of an siRNA duplex have the ability and opportunity to enter into the RISC complex for effective RNAi if their target mRNAs are accessible.

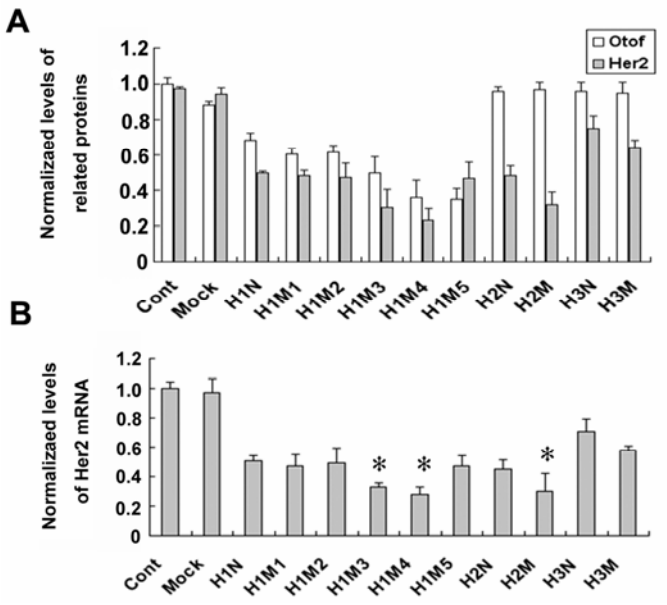

Fig. 2. The sense strand can enhance the efficacy of siRNAs to different extents. A – When SK-BR-3 cells were at 70-80% confluence, cells were transfected by siRNAs at 100 ng, while 100 ng mock siRNA was introduced into the cells as a control. A reduction in the protein levels of Her2 (solid bars) and Otoferlin (empty bars) was determined by Western blotting analysis 36 hours after transfection. The expression levels were normalized by the percentage of the protein levels in siRNA-transfected cells relative to their levels in control cells. B – Real Time-PCR analysis on the expression levels of Her2 mRNAs under various conditions were carried out and normalized to that of GAPDH, and the resultant expression levels in different cases are normalized to their levels in the control. The data is the averages of at least three independent determinations. Error bars indicate standard deviations. *P < 0.01; H1M3 or H1M4 compared with H1N, and H2M compared with H2N, respectively.

The fork terminus enhances the silencing efficacy of siRNAs with high C/G contents

Some findings demonstrate that the RISC complex has a special capacity to select and unwind a hermodynamically weak end of siRNA. To examine whether this occurs with an siRNA duplex with a high C/G content (> 53%), we designed seven fork-siRNAs by introducing one to four mutated bases into the 3’ or 5’ end of siRNAs. After the transfection of different siRNAs for 24 h, Real

Time-PCR analysis demonstrated that H1M3-siR and H1M4-siR greatly conferred a further enhancement in RNAi activity compared with the H1N-siR in silencing Her2 mRNA. However, H1M1-siR and H1M2-siR slightly increased the efficacy of target gene silencing (Fig. 2). Careful observation of these mutated sequences revealed that the number and position of the mutated nucleotide could exert some influence on the silencing efficiency. For example, the H1M1-siR, H1M2-siR, H1M3-siR and H1M4-siR gradually increased the Her2 inhibition with the shift in the position of the mutant nucleotides from the 15^{th} to19^{th} position in the sense strand and the increase in mutant number. This implies that the mismatches occurring in the 18^{th} and 19^{th} positions may play a much stronger role in facilitating guide-strand assembly than those in the 15^{th} and 16^{th} positions. These results were further supported by the observations on the effects of two other siRNAs, H2M-siR and H3M-siR (Fig. 2B). On the other hand, H1M5-siR with two mutations in the 3' terminus of its antisense strand also displayed an increase in the silencing of target genes such as Otoferlin and Calpain. This provided further evidence to support our view that both strands of siRNA can be functional, and that the efficacy of their entering into RISC can be enhanced by the introduction of mutations into different ends.

Figs 1 and 2 illustrated that H1M4-siR not only enhanced the effectiveness of its antisense strand, but surprisingly also greatly improved the efficacy of its sense strand in suppressing Otoferlin mRNA, owing to an increase in base-pairings. This demonstrates that the sense strand can still be assembled into RISC, even under disadvantageous conditions, and can exert a role in RNAi. This suggests that lowering the stability of one end greatly does not completely block the effectiveness of the other strand integrated into the RISC. Further investigation also revealed that the changes in both the Her2 and Otoferlin gene levels induced by H1M4-siR and H1N-siR duplexes were statistically significant ($P < 0.01$), suggesting that, besides the end stability, additional factors such as the extent of base-pairings and accessibility to the local mRNA structure may play roles in gene silencing (Fig. 2B).

Accessibility to local mRNA structure influences the silencing efficacy of siRNA

The RNAi process may be obstructed either physically, by inaccessible target mRNA structure, or chemically, by the ineffective unwinding of the target mRNA or the imperfect pairing of the corresponding mRNA [25, 31, 32]. To verify the influence of secondary structures of the target mRNA on the efficacy of unique siRNAs, the SYBR® Green based Q-PCR method was used. The threshold cycle (*C*t), which indicates the significant increase in fluorescence level, was compared between the control and experimental groups (Fig. 3A). We investigated the effects of wild and mutated types of Her2-siRNA on two different mRNAs. The same sense strand of H1N-siR induced a slight decrease in the levels of both Calpain and Otoferlin mRNAs, even though the former is

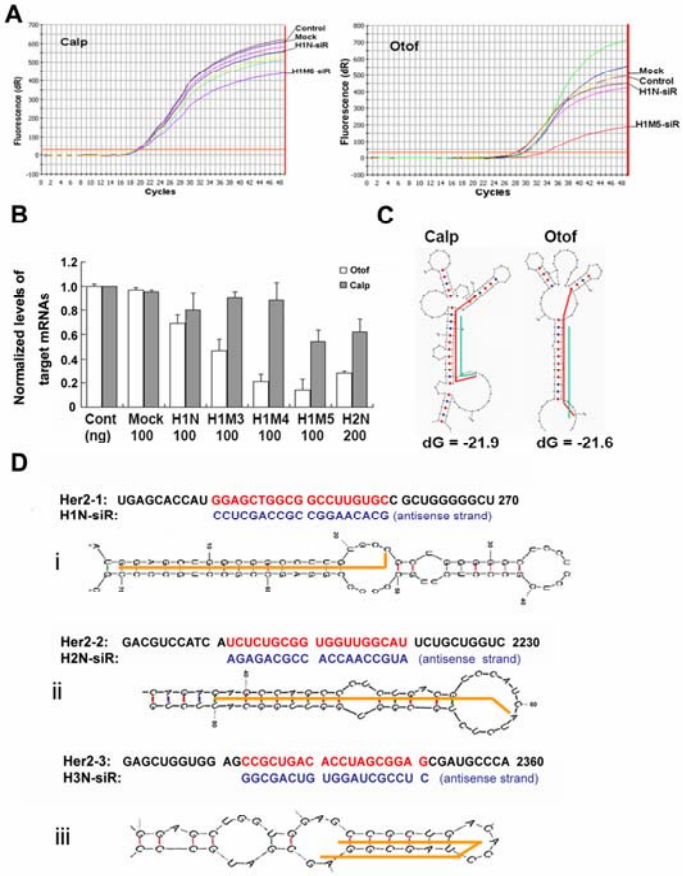

Fig. 3. The local secondary structures of target mRNAs may influence the efficacy of corresponding siRNAs in gene silencing. A – When SK-BR-3 cells were at 70-80% confluence, cells were transfected by different siRNAs at 100 ng or 200 ng, while 100 ng mock siRNA was introduced into the cells as a control. Real Time-PCR amplification plots of Calpain5 mRNA isolated from cells transfected with different siRNAs and control cells 24 hours after transfection, and Otoferlin mRNA treated with different siRNAs and control cells 24 hours following treatment. B – The expression levels of target genes were examined by means of real-time PCR using the synthesized cDNAs as templates. The expression levels of either Otoferlin or Calpain5 are normalized to that of GAPDH, and the resultant expression levels in different cases are normalized to the expression levels determined in the control case. The results are representative experiments in triplicate and are displayed as means and S.D. C – Calp and Otof respectively represent the local structures of Calpain and Otoferlin target mRNAs. All the local structures were folded using the Mfold. The outputs of the secondary structure prediction are shown in the graph. Relevant regions of structures with the lowest free energy are shown. The target sequences for the full or partial matches with guide strands are respectively marked by a red or green line. D – The sequence alignment of Her2 mRNA and different siRNAs (H1N-siR, H2N-siR and H3N-siR) as well as the secondary structures predicted for different local regions (Her2-1, Her2-2 and Her2-3) of Her2 are shown in the graph. The number in the Her2 sequence indicates the position of the last nucleotide. The yellow line in the foldback structures represents the target region of the siRNA.

perfectly complementary, and the latter is partially base pairing. Subsequently, H1M3-siR was employed to further investigate this case, because H1M3-siR has 17 of 19 fully matched bases to both the target genes, Calpain5 and Otoferlin. Unexpectedly, the RT-qPCR results revealed a significant difference between the Otoferlin and Calpain5 levels (Fig. 3B). This observation was further supported by the fact that, when the sequence of the sense strand gradually became fully complementary to its target region of the Otoferlin gene, H1M4-siR could yield a remarkable reduction (by about 85%) in the level of Otoferlin mRNA. By contrast, there was an increase in the expression of Calpain mRNA when the base pairing between H1M3-siR or H1M4-siR and the Calpain target was reduced from 19 nt to 15 nt. Moreover, local free energies (dG_{loc}) were calculated for the structural stability. We found that the local free energies of Calpain5 and Otoferlin were very similar (Fig. 3C). Careful analysis on the local structure of Calpain indicated that the poor exposure of the target region was due to several bulges around the target sequence, which possibly form a kinetic trap to conceal the target sequence in the local mRNA folding. By contrast, the Otoferlin target had a good exposure of the target sequence to the siRNA. Moreover, the secondary structure of the local regions of Her2 targeted by H1N-siR, H2N-siR and H3N-siR was compared. The results showed that the target region of H3N-siR was a hairpin structure that was not easily accessible (Fig. 3Diii) while those regions targeted by H1N-siR and H2N-siRNA displayed a similar stem output. As expected, the efficacy of H3N-siR was the lowest among these siRNAs (Fig. 2), while the efficacies of H1N-siR and H2N-siR were similar to each other (Fig. 3Di and 3Dii). The findings suggest that the poor exposure of the target sequence within the mRNA performs an important role. Although it is known that structure predictions have strong limitations when long RNAs are concerned, mFOLD still is a useful method for the calculation of local free energy and the identification of stable local structure (See the Materials and Methods section). Collectively, this experimental evidence supports the view that the silencing efficacy of siRNA is determined by both the siRNA sequence itself and the local structure of the target mRNA.

Multi-factors cooperatively regulate the silencing efficacy of siRNA

To re-examine to what extent the base pairing of the target region affects the efficacy of siRNA in gene silencing, we tested what roles mismatches at the ends played in the suppression process. It was notable that in this system, single to multiple base mutations introduced into the 3' end of siRNA duplexes could enhance the activity of the sense strand of the H1N-siR duplex in silencing Otoferlin. In comparison with the effectiveness of H1N-siR and H1M3-siR or H1M4-siR, RT-qPCR results depicted that the duplexes H1M3-siR and H1M4-siR induced 2- to 3-fold more reduction in sequence-specific *Otoferlin* gene silencing (Fig. 3B) than H1N-siR. Increased pairings between H2M3-siR or H2M4-siR and the Otoferlin target might facilitate the binding to the target region and formation of the A-form helix for RNAi catalysis, implying that the

chemical contact between the guide strand and the target region of the mRNA is more important than physical accessibility. On the other hand, when two nucleotide mismatches were introduced at the 3'-end of the antisense of H1N-siR, the results indicated that these two mismatches in the 3' terminus of the antisense strand of H1M5-siR also increased the efficacy of the target Otoferlin and Calpain degradation (Figs 1 and 2). Quantitative analysis indicated that this mutated siRNA could greatly enhance the efficacy in suppressing the expression of Calpain5 and Otoferlin genes, respectively to a level about 20% and 50% higher than H1N-siR. Careful comparison of the efficacy between H1M3-siR or H1M4-siR, and H1N-siR revealed that a one-fold increase in the concentration of siRNA was almost equivalent to the enhancement of two base pairing matches (Fig. 2). These results further demonstrate that the sequence context of an siRNA is not a sufficient dominant in determining the efficacy of that siRNA, and that other factors such as the local structures of target mRNAs and the type of siRNA are also important.

The target localization regulates modes of gene silencing

Some observations indicated that although Ago1 through Ago4 bind small RNAs regardless of their sequence, only Ago2-containing miRNPs were able to guide the cleavage of the complementary target. To investigate the relationship

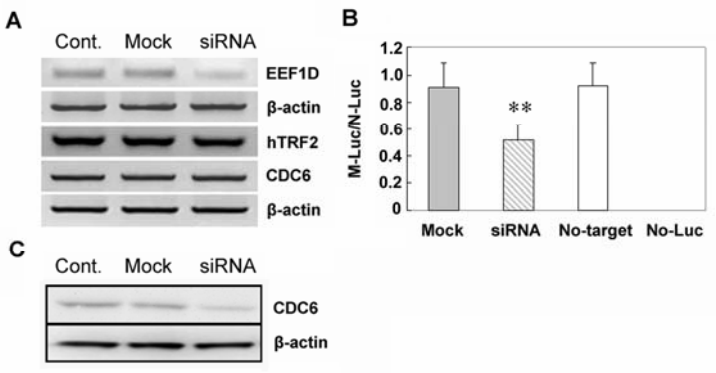

Fig. 4. The same siRNA can silence target mRNAs in different modes of action through the interaction with different target regions such as 5'- and 3'-UTRs. A – When SK-BR-3 cells were at 70-80% confluence, cells were transfected by siRNAs at 100 ng, while mock siRNA (100 ng) was introduced into the cells as a control. RT-PCR analysis was employed. B – The siRNA (500 ng) together with 2 ug pRL-TK reporter plasmids with or without a target sequence of the siRNA were cotransfected into SK-BR-3 cells, while mock siRNA (500 ng) together with 2 ug pRL-TK reporter plasmids with or without the target sequence were used as a control. 24 h after transfection, a dual luciferase assay was carried out. Relative levels of the expressed luciferase activity under various conditions were determined and normalized to their levels in the mock control. The data is the averages of at least three independent determinations. Error bars indicate standard deviations. **P < 0.01; S-siRNA compared with the control group. C – The levels of CDC6 protein from different cases were analyzed by Western blotting. The Western blot was stripped and re-probed with actin antibody to check for equal loading of total protein.

between the target localization and siRNA modes of action, we designed a special siRNA with a high C/G content in both ends, S-siRNA. As this siRNA is homogenous to Alu sequences, it can silence many different mRNAs with the Alu element in the 5'UTR or the 3'UTR region (Tab. 3). For example, EEF1D encodes eukaryotic translation elongation factor 1 delta. The S-siRNA is extensively complementary to the target region of the 5'UTR of EEF1D. RT-PCR illustrated that it resulted in a remarkable level of gene silencing, as shown in Fig. 4A. More interestingly, we found that this siRNA also targeted hTRF2 and CDC6 mRNAs through the interaction with their 3'UTR regions. Inconceivably, this S-siRNA did not result in an observable decrease in the steady-state mRNA levels, although it is fully complementary to the target sequences of hTRF2 and has only one mismatched nucleotide with the CDC6 mRNA (Fig. 4A). Subsequently, the Luciferase activity assay was used to better validate the efficacy of the S-siRNA inhibitor using a Luciferase reporter bearing the S-siRNA target sequence cloned into its 3'-UTR. Quantitative experiments revealed that this S-siRNA gave a significant decrease in Luciferase expression (by 50%), while the mock siRNA did not cause an obvious reduction (Fig. 4B). This observation was further supported by the western blotting analysis, which showed that the level of CDC6 protein was down-regulated in the s-siRNA treated group but not in the mock and control cases (Fig. 4C).

DISCUSSION

This study investigated the fundamental factors determining the efficacy of siRNAs in silencing endogenous genes by employing a model with the same siRNA and different endogenous target mRNAs, rather than the method used in other studies, with different siRNAs targeting the same mRNA. The evidence showed that the access to the target mRNA, the sequence context of the siRNA, the complementary extent of the guide strand and target region, the siRNA forms and the action localization of the siRNA are the five essential features in the process of gene suppression mediated by siRNAs. Based on our findings and experimental results from other laboratories, several conclusions about the efficacy of siRNAs can be drawn. First, the introduction of mutated nucleotides to the 3' terminus of the sense strand does indeed enhance the silencing efficacy of its antisense strand. Second, the local structures of mRNAs may impede the access of siRNAs to their targets. Third, siRNA with a high CG content targeting the 5'- or 3'-UTR region of mRNAs can give different modes of gene silencing. And fourth, the target selection may determine whether the guide strand is maintained in RISCs or discarded. In addition, our data indicates that some siRNAs may inhibit gene expression in a translational repression or slow cleavage manner, even though complete base-pairings occur between the siRNA and its target mRNA.

Several lines of evidence have shown that helicase activity may be affected by unwinding of the unstable 5' antisense terminus. Similarly, some studies indicate

that fork-siRNA duplexes can promote the antisense strand of siRNA duplexes to enter into an RISC complex [33, 34]. Although the thermodynamic properties of siRNA play a key role in unwinding the duplex and making strands enter into RISC [17], some studies have shown that the direction of the Dicer-2/R2D2 processing may influence strand selection [35, 36]. More importantly, a recent study revealed that Ago2 prefers to capture the double-stranded siRNA and then chop the siRNA passenger strand, thereby taking the single-stranded guide [37]. We hypothesize that fork-siRNAs facilitate the recognition and capture of the corresponding antisense-strand of the siRNA duplex by the PIWI domain in Ago2. The involvement of the PIWI domain in RISC assembly is supported by previous observations [38]. Furthermore, our findings indicate that both stands of siRNAs possess the ability to enter into RISC and effectively perform their silencing function, although there is a difference in the amounts of sense or antisense strands maintained in RISCs. Thus, the end stability of the siRNA may influence how easily the strands of that siRNA are unwound, and how effectively one strand is selected as the functional strand in RISCs, while the survival of these strands in cells may finally depend on the selection pressure exerted by the mRNA targets, even though they can initially bind to the RISC complex regardless of the existence of their targets. The easier the guide strands find their targets, the more survive. Otherwise, they are discarded and degraded (Fig. 5). This view is consistent with the observation that there are more sense strands incorporated into RISC in the presence of their target RNA [15]. In addition, some investigations also indicate that fork-siRNAs can reduce off-target effects besides the ability to enhance RNAi activity [15, 33]. However, our results suggest that no mutations introduced into a given siRNA will alleviate all off-target effects.

We found that the main difference in the activity of the same siRNAs in gene silencing is partially due to their accessibility to different target mRNAs. The secondary structure of mRNA can form diverse obstacles that impede the access of siRNAs and further interaction between siRNAs and their target mRNAs. The access of siRNAs to their target mRNAs can be defined in terms of physical and chemical contacts. The first step is related to the physical contact of the siRNA molecule to its target RNA. This is dependent on the accessible status and extent of the local region of the target RNA. These physical barricades can be divided to at least four types: a hairpin structure, a v-type structure [25], a hidden structure caused by a large bulge at the complementary sequence, and a combination of these types. During the interaction process of the guide strand and its target sequence, chemical blockage plays an important role. It embraces excessively stable intramolecular structures such as high G/C contents and imperfect base-parings. As shown by our research results (Figs 3 and 5), target accessibility and interaction are of importance in selecting and maintaining an effective siRNA strand in RISC [39]. Our observation is consistent with other findings. Bohula *et al.* [40] discovered that the efficacy of siRNAs to induce gene silencing correlated closely with the accessibility of the target sequence

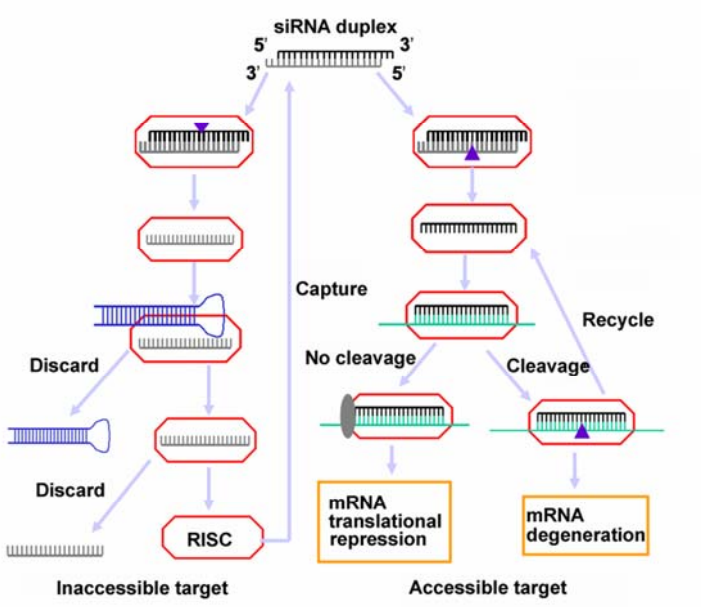

Fig. 5. A model for the interaction between RISCs, siRNA elements, target mRNAs and other factors for gene silencing. RISCs are schematically depicted, and the two strands of siRNA are respectively colored gray and black. siRNAs are initially loaded into RISCs as duplexes, and then the passenger strand of the siRNA is sliced and discarded, leaving the siRNA guide strand bound stably to the RISC. Before RISCs containing the sense strand or antisense strand interact chemically with the corresponding target mRNA, they should first gain physical access to the homogenous region of the target mRNA. The RISC accessible to its target mRNA can display a relevant function, such as translational repression, or mRNA degradation. After releasing the old target mRNA, it can bind to a new one for another recycle. However, an RISC inaccessible to the target would discard that guide strand, and then rebind to a new siRNA. Thus, target mRNAs would create a selection pressure to RISCs, resulting in the survival of those strands that can interact with target mRNAs.

within the transcript, suggesting that the secondary structure of the target mRNA was a necessary factor in determining the potency of siRNAs. Lou and Chang [41] indicated that the formation of a hairpin structure within the siRNA target region could greatly reduce the efficacy of siRNAs. All these results support the view that intramolecular folding of the mRNAs renders most transcripts inaccessible to binding of the complementary nucleic acids [40, 42]. Recently, some bioinformatics data showed that, if accessibility predictions are carried out, the random selection of siRNAs is better for gene silencing. In addition, this random prediction gives fewer false negatives than GC-content prediction does [25]. Therefore, the local structure may play a role in determining which guide strands should be maintained in RISCs, and which should be discarded.

One current model of RNA silencing suggests that the different sequence context of small RNAs can recognize and bind to different Ago proteins, producing differential biological effects [43, 44]. Eight members of the Argonaute protein family have been identified in human cells. Given that Ago1, Ago3 and Ago4 do not direct target degradation, only Ago2 guides target RNA cleavage [11]. Here, we find that the same siRNA targeting the Alu element within 3'UTR or 5'UTR can direct different modes of action on mRNA repression, even though the extent of sequence-complementarity between S-siRNA and its target region is the same. The S-siRNA targeting 3'UTR regions may predominantly function as slow cleavage triggers or translational repressors, while the same S-siRNA is competent to yield detectable cleavage via interaction with the 5'UTR regions. It is reasonable to hypothesize that Ago2 complexes have a dual function in guiding both cleavage and translational repression according to the complementary types with target mRNA, the local secondary structure of mRNA targets, and additional inhibitory factors in the 3'-end [45, 46]. Some inhibitory proteins different in the 5' and 3' terminuses may be involved in the interaction with RISCs, which in turn modulate the action modes of RISC, cleavage or repression [47]. This idea is strongly supported by the siRNAs having different functions, such as DNA methylation [48], mRNA cleavage, or translational repression, depending on the action site between the small RNAs and their targets. However, it remains unsolved how the RISC-assembly machinery decides to take which modes of silence: translation repression, slow degradation or fast cleavage.

Taken together, this investigation brings into focus the question of which factors determine the efficacy of siRNAs in gene silencing. We deeply explored the possible working mechanisms that manipulate the interaction of siRNA and its target mRNA, and provided important indications for the design of effective sRNAs. We recommend that the following points should be carefully considered in order to obtain efficient and specific siRNAs: (i) the sense strand of a siRNA duplex with partial complementarity to target mRNAs may be functional in inhibiting target genes; (ii) fork-siRNAs can be used to enhance RNAi activity by speeding up the entry of the guide strand into the RISC, and to remove the off-target effect of the sense strand by introducing base mutations [15, 49]; and (iii) the local structures should be tested to ensure the exposure of the target sequence. In addition, our data also suggests that there are different modes of action for the same siRNA, implying that other proteins are involved in regulating the modes of action of RISC. The molecular mechanisms require further study.

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