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Molecules and Cells

Dual-Target Gene Silencing by Using Long, Synthetic siRNA Duplexes without Triggering Antiviral Responses

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Chemically synthesized small interfering RNAs (siRNAs) can specifically knock-down expression of target genes via RNA interference (RNAi) pathway. To date, the length of synthetic siRNA duplex has been strictly maintained less than 30 bp, because an early study suggested that double-stranded RNAs (dsRNAs) longer than 30 bp could not trigger specific gene silencing due to the induction of non-specific antiviral interferon responses. Contrary to the current belief, here we show that synthetic dsRNA as long as 38 bp can result in specific target gene silencing without non-specific antiviral responses. Using this longer duplex structure, we have generated dsRNAs, which can simultaneously knock-down expression of two target genes (termed as dual-target siRNAs or dsiRNAs). Our results thus demonstrate the structural flexibility of gene silencing siRNAs, and provide a starting point to construct multifunctional RNA structures. The dsiRNAs could be utilized to develop a novel therapeutic gene silencing strategy against diseases with multiple gene alternations such as viral infection and cancer.

INTRODUCTION

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism conserved in diverse eukaryotes. When double-strand (ds) RNA is introduced into cells, it is cleaved by Dicer, an RNase III enzyme, into 21 to 23 nucleotides (nts) with 2 nt 3′ overhangs (Elbashir et al., 2001a; Zamore et al., 2000). The resulting small interfering RNAs (siRNAs) are recognized by RNA-Induced Silencing Complex (RISC)-Loading Complex (RLC), and then loaded onto RISC. An active RISC complex that harbors the antisense strand of siRNA recognizes and cleaves the target mRNAs containing complementary sequences (Khvorova et al., 2003; Schwarz et al., 2003).

Originally, dsRNA-triggered RNAi was discovered in invertebrates such as *C. elegans* (Fire et al., 1998) and *D. melanogaster* (Kennerdell and Carthew, 1998). In contrast, RNAi-mediated gene silencing in mammalian cells showed limited success due to the induction of antiviral responses and subsequent non-specific gene suppression triggered by long dsRNAs (Caplen et al., 2000; Ui-Tei et al., 2000). However, Elbashir et al. (2001b) demonstrated that specific gene silencing in mammalian cells could be achieved by reducing the length of duplex to 19 to 21 bp, which circumvents the surveillance mechanism of mammalian antiviral innate immune responses. They also suggested that one could not execute specific gene silencing by using dsRNAs longer than 30 bp (Elbashir et al., 2001b), due to the activation of antiviral interferon responses (Stark et al., 1998). Duplex length of the recently developed 27 bp-long Dicer-substrate siRNAs (Kim et al., 2005) also falls within this range.

Because of the efficient and specific silencing of target gene expression, drug development using RNAi technology has grown rapidly and is expected to be one of the most promising future therapeutics for many diseases, including viral infection and cancer. RNAi-mediated inhibition of viral replication has been achieved transiently by synthetic small interfering RNA (siRNA) or stably by short hairpin RNA (shRNA) expression constructs (Jacque et al., 2002; Nishitsuji et al., 2004; Novina et al., 2002). However, single gene silencing by RNAi has limitations, because viruses eventually escape from RNAi by rapid mutations in their genomes (Boden et al., 2003; Das et al., 2004; Westerhout et al., 2005). Simultaneous introduction of two or more siRNAs/shRNAs targeting different viral genomic positions overcame this problem (Konstantinova et al., 2006; Watanabe et al., 2006).

In addition to the antiviral therapeutics, RNAi technology is also being developed for anti-cancer therapeutics. Again, compared with the single gene silencing, simultaneous silencing of multiple genes involved in multiple pathways critical for cancer cell growth and survival should be beneficial as it potentiates the anti-neoplastic effects and at the same time reduces the chance of developing drug resistance (Menendez et al., 2004). Therefore, development of an efficient method to execute simultaneous silencing of multiple genes by RNAi, which is termed as combinatorial RNAi (Grimm and Kay, 2007), is highly desired.

While a number of combinatorial RNAi strategies have been developed for shRNAs (Konstantinova et al., 2006; ter Brake et

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al., 2006; Watanabe et al., 2006) and long hairpin RNAs (lhRNAs) (Liu et al., 2007; Sano et al., 2008), few examples of multi-target siRNA structures have been reported. A recent study described dual-target silencing siRNA structures comprised of partially complimentary antisense strands (Hossbach et al., 2006); however, they suffered from incomplete annealing due to the presence of multiple mismatches between the strands. Another study (Khaled et al., 2005) utilized the phi29 RNA backbone to build RNA nanostructures with three different functional positions, and two to three siRNAs could be simultaneously introduced into cells. However, the RNA backbone used is too long to be chemically synthesized, limiting its practical utility. Therefore, development of RNA structures harboring multiple siRNAs that is amenable to chemical synthesis is of great importance.

In this study, we attempted to develop chemically synthesized RNA structures, which can efficiently and simultaneously knock-down two different target genes. Surprisingly, and contrary to the current belief, 34 to 38 bp long dsRNA duplexes constructed by a simple fusion of two 17 or 19 bp-long siRNA units efficiently silenced two different target genes without triggering significant non-specific antiviral interferon responses. We termed these structures as dual-target gene silencing siRNAs (dsiRNAs).

MATERIALS AND METHODS

siRNAs

Chemically synthesized RNAs were purchased from Bioneer and annealed according to the manufacture's protocol. Sequences and structures of siRNAs used in experiments are shown in figures.

Cell culture and siRNA transfection

HeLa or T98G cells were cultured in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in 12-well plates 24 h before transfection at 30-40% confluency in complete medium without antibiotics. All transfections were performed using the Lipofectamine 2000 reagent following the manufacturer's protocol (Invitrogen). 10 nM concentration of siRNAs were used for all experiments.

Quantitative RT-PCR

Total RNAs were extracted from cell lysates using the Tri-reagent kit (Ambion). Total RNA (1 μg) was used as a template for cDNA synthesis, which was performed with the ImProm-II™ Reverse Transcription System (Promega) according to the manufacture's protocol. Aliquots (1/20) of the cDNA reactions were analyzed by quantitative RT-PCR using a Rotor-Gene 3000 PCR machine (Corbett Research) according to the manufacture's protocol. Gene-specific primers for target genes were mixed separately with Power SYBR Green PCR Master Mix (Applied Biosystems) containing the cDNA to be analyzed, and the mixture was placed into 0.1-ml tubes. Samples were tested in duplicate, and the data obtained were analyzed with Rotor-Gene 6 software (Corbett Research). The primer sequences for each gene were:

TIG3-forward 5′-AGA TTT TCC GCC TTG GCT AT-3′
TIG3-reverse 5′-TTT CAC CTC TGC ACT GTT GC-3′
Lamin-forward 5′-CCG AGT CTG AAG AGG TGG TC-3′
Lamin-reverse 5′-AGG TCA CCC TCC TTC TTG GT-3′
GAPDH-forward 5′-GAG TCA ACG GAT TTG GTC GT-3′
GAPDH-reverse 5′-GAC AAG CTT CCC GTT CTC AG-3′
OASIS-forward 5′-CCG GAG GAA AAT CAA GAA CA-3′
OASIS-reverse 5′-ATT CTC CAG GGT CTC CAC CT-3′
DBP-forward 5′-CCT CGA AGA CAT CGC TTC TC-3′
DBP-reverse 5′-GCA CCG ATA TCT GGT TCT CC-3′
OAS2-forward: 5′-TCA GAA GAG AAG CCA ACG TGA-3′;
OAS2-reverse: 5′-CGG AGA CAG CGA GGG TAA AT-3′
IFN-β-forward: 5′-AGA AGT CTG CAC CTG AAA AGA TAT T-3′
IFN-β-reverse: 5′-TGT ACT CCT TGG CCT TCA GGT AA-3′
IFIT1-forward: 5′-AAA AGC CCA CAT TTG AGG TG-3′
IFIT1-reverse: 5′-GAA ATT CCT GAA ACC GAC CA -3′

Northern blot hybridization

Total RNA (15 μg) was resolved by agarose/formaldehyde gel electrophoresis, transferred by capillary method on to a nylon membrane, and hybridized to specific radiolabeled DNA probes, which were prepared from the HeLa cell cDNA by the random hexamer labeling kit (invitrogen) and [α-^{32}P] dCTP (New England Nuclear). Washed membranes were exposed to imaging plate (BAS-IP MS 2040, Fuji film) and analyzed on a phosphoimage analyzer (FLA-2000, Fuji photo film Co.LTD). Image analysis software (Multi Gauge version 3.0) was used to quantify the signals for each lane on the blot.

Native gel electrophoresis

An aliquot of each siRNA was separated in a 15% (w/v) nondenaturing polyacrylamide gel, stained with EtBr, and visualized by UV transillumination.

Luciferase reporter assay

Reporter plasmids for luciferase assay were described previously (Chang et al., 2009). 24 h after transfection, cells were lysed with the Passive lysis buffer of the Dual-luciferase Reporter Assay System (Promega). Using a Victor3 plate reader (PerkinElmer), luciferase activity was measured in 20 μl of each of the cell extracts using 100 μl of substrates for both firefly and *Renilla* luciferase.

5′-RACE assay

20 h after siRNA transfection into HeLa cells, total RNAs were extracted using Tri-reagent kit (Ambion). 2 μg of total RNAs from each siRNA transfected cells were ligated to 0.25 μg of GeneRacer RNA oligo, and then reverse-transcribed using GeneRacer oligo(dT) and SuperScript™ III RT kit (Invitrogen). The RT products were PCR-amplified using gene specific primers. The resulting PCR products were cloned into T&A vector (RBC) and sequenced using M13 forward primer. The gene specific primer sequences for RACE PCR were:

TIG3 Gene specifie 3′ primer :
5′-GGGGCAGATGGCTGTTTATTGATCC -3′
TIG3 Gene specific 3′ nested primer :
5′-ACTTTTGCCAGCGAGAGAGGGAAAC-3′
Lamin Gene specific 3′ primer :
5′-CCAGTGAGTCCTCCAGGTCTCGAAG-3′
Lamin Gene specific 3′ nested primer :
5′-CCTGGCATTGTCCAGCTTGGCAGA-3′

RESULTS

Dual-target gene silencing siRNAs (dsiRNAs) can be generated by combination of two siRNA duplexes

Previously, we made a heterologous RNA duplex extension to a 19 bp siRNA duplex to yield 30 bp-long dsRNA (Chang et al., 2007). This long duplex efficiently silenced target gene without non-specific interferon responses. Based on this finding, we attempted to build a dsRNA structure which can efficiently and

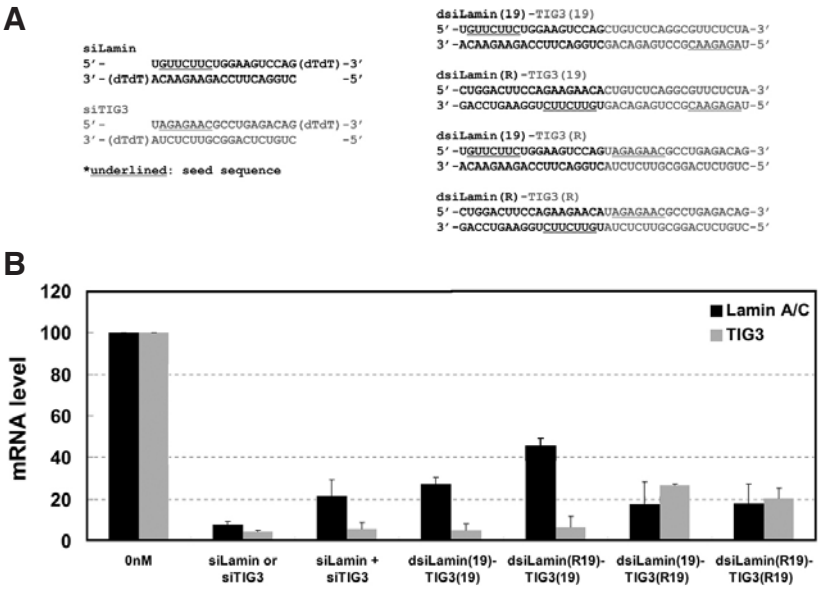

Fig. 1. Structures and gene silencing activities of 38 bp-long dsiRNAs. (A) Structures of dsiRNAs that target lamin A/C and TIG3 mRNAs. 38 bp dsiRNAs with diverse directionalities were constructed by annealing two 38 nt ssRNAs. (B) Activities of dsiRNAs that target Lamin A/C and TIG3 mRNAs. siRNAs or dsiRNAs (10 nM each) were transfected into HeLa cells using Lipofectamine2000, and 24 h after transfection, Lamin A/C and TIG3 mRNA levels were analyzed by quantitative RT-PCR. The mRNA levels of Lamin A/C or TIG3 mRNA relative to GAPDH is plotted on the y-axis. All data in the graph represent means+standard deviation (SD) values of three independent experiments.

simultaneously silence the expression of two different genes [referred to as dual-target silencing siRNA (dsiRNA)]. We first designed 38 bp duplexes which are simple fusion of two 19 bp siRNA duplexes, siLamin and siTIG3, each targeting Lamin A/C and TIG3 mRNAs, respectively (Fig. 1A). Fusion of two 19 bp siRNA duplexes with possible combinations yielded four different 38 bp duplexes. Each 38 bp dsiRNA structure was built by annealing two complementary 38 nt single-stranded (ss) RNAs. dsiLamin(19)-TIG3(19) structure was designed to have 5′-end of antisense strands of both siRNAs headed towards outside of the RNA duplex; dsiLamin(R19)-TIG3(19) structure was designed to have 3′-end of siLamin antisense strand and 5′-end of siTIG3 antisense toward outside of the RNA duplex; dsiLamin(19)-TIG3(R19) structure has 5′-end of siLamin antisense strand and 3′-end of siTIG3 antisense strand toward outside of the RNA duplex; finally, dsiLamin(R19)-TIG3(R19) structure was designed to have 3′-end of antisense strands of both siRNAs toward outside of the RNA duplex. All 38 bp dsiRNA duplexes were correctly annealed (Fig. S1)

To evaluate the gene silencing efficiency of these dsiRNA structures, we transfected HeLa cells with the dsiRNA constructs using Lipofectamine2000. The gene silencing activities of dsiRNAs were compared with those of siLamin, siTIG3, and the mixture of siLamin and siTIG3. 24 h after transfection, Lamin A/C and TIG3 mRNA levels were measured using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Compared with individual siRNA transfection, mixture of siLamin and siTIG3 resulted in similar reduction of TIG3 mRNA (Fig. 1B); however, the silencing of LaminA/C expression was somewhat reduced (Fig. 1B), which is likely due to the competition between siLamin and siTIG3 (Yoo et al., 2007). We then compared the target gene silencing by various dsiRNAs with that by the siLamin/siTIG3 mixture. Out of these four 38 bp dsiRNA constructs, only dsiLamin(19)-TIG3(19) showed comparable silencing of both genes (Fig. 1B). Similar results were obtained when we transfected this dsiRNA into T98G cells (Fig. S2). DsiLamin(R19)-TIG3(19) maintained comparable silencing activity for TIG3 gene, but reduced silencing activity for Lamin A/C gene (Fig. 1B). The opposite was true for dsiLamin(19)-TIG3(R19), with good silencing efficiency for LaminA/C gene, but reduced silencing activity for TIG3 gene (Fig. 1B). Another dsiRNA structure, dsiLamin(R19)-TIG3(R19), also failed to achieve efficient silencing for both targets (Fig. 1B). These results demonstrate the importance of directionality when dsiRNAs are made by combination of two 19 bp siRNA units. Only the dsiRNA structure, in which 5′-end of antisense strand of both 19 bp siRNA units are headed toward outside, maintains activities of both siRNAs. This directionality is consistent with our previous observations (Chang et al., 2007), where we demonstrated that a heterologous duplex extension at the 5′-end of the antisense strand resulted in loss of gene silencing activity of the siRNA; however, duplex extension at the 3′-end of the antisense strand did not have any effect.

We then tested whether we could apply this directional conjoining rule between two siRNAs to build other dsiRNAs. dsiOASIS (19)-TIG3(19), dsiLamin(19)-DBP(19), and dsiDBP(19)-TIG3(19) were designed to have the 5′-end of antisense strands of 19 bp siRNA units headed toward outside of dsiRNAs (Fig. S3A), and the gene silencing efficiency of each dsiRNA was compared with that of its corresponding siRNA mixture in HeLa cells. dsiOASIS(19)-TIG3(19), dsiLamin(19)-DBP(19), and dsiDBP(19)-TIG3 (19) efficiently inhibited both target mRNAs, and the silencing efficiencies were comparable to those of 19 bp siRNA mixtures (Figs. S3B-S3D). Therefore, All dsiRNAs constructed on the basis of our connection rule successfully knocked down two target genes simultaneously.

DsiRNA shorter than 38 bp can efficiently silence both target genes

An early study (Elbashir et al., 2001b) identified the 19 bp RNA duplex as the most efficient trigger of RNAi in mammalian cells. However, the importance of siRNA sequence within the duplex for target mRNA recognition is position dependent, with sequences near the 5′-end of the antisense strand (i.e. the seed sequence) being most important (Birmingham et al., 2006). Therefore, when 19 bp RNA duplex is used as an RNAi trigger, partial complementarity of siRNA to mRNA is often sufficient to execute target gene silencing (Jackson et al., 2003; 2006). From this, we hypothesized that, because our dsiRNAs have sufficient length to be recognized by Dicer (Kim et al., 2005), we might be able to achieve efficient gene silencing with a dsiRNA with each siRNA unit length shorter than 19 bp. To test

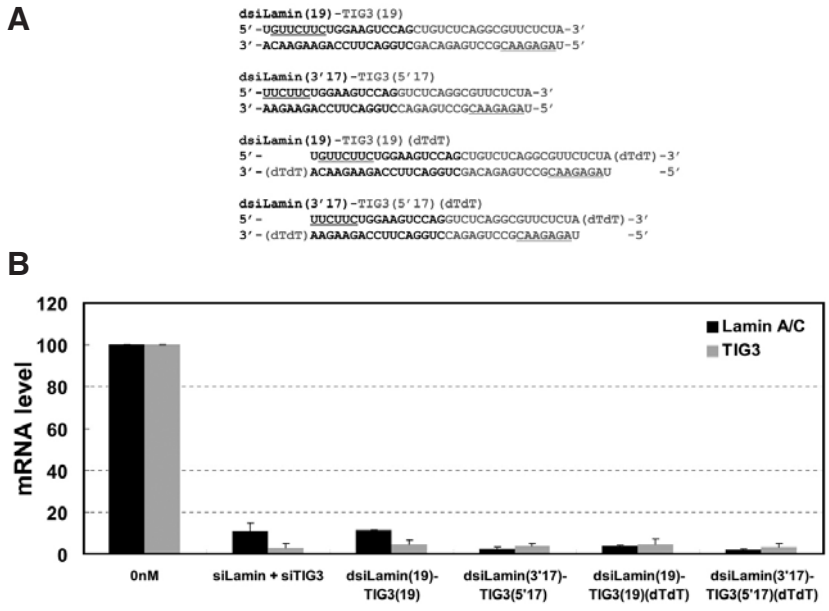

Fig. 2. Effects of duplex length and overhang structure on dsiRNA activity. (A) Structures of 38 and 34 bp dsiRNAs with or without overhangs targeting Lamin A/C and TIG3 mRNAs. (B) Activities of diverse structures of dsiRNAs (10 nM) that target Lamin A/C and TIG3 mRNAs. See the legend of Fig. 1B for details.

this, we synthesized several shorter dsiRNAs of 30-34 bp duplexes targeting Lamin and TIG3 mRNAs. We found that one 34 bp-long dsiRNA, dsiLamin(3′17)-TIG3(5′17), which contains 17 bp from 3′-end of siLamin antisense and 17 bp from 5′-end of siTIG3 antisense, executed efficient silencing of both target genes, in spite of their length being shorter than 38 bp dsiRNAs (Figs. 2A and 2B). Surprisingly, the silencing efficiency of the 34 bp-long dsiRNA on both target genes was better than both the mixture of two 19 bp siRNAs and the 38 bp-long dsiRNA. Similar results were obtained when we transfected the 34 bp-long dsiRNA into T98G cells (Fig. S2). We also confirmed efficient and specific gene silencing by dsiRNAs using Northern blot analysis (Fig. S4).

A previous study reported that the 2 nt-overhang at the 3′-end of both strands is important for the siRNA activity (Elbashir et al., 2001c). Because the 34 and 38 bp dsiRNAs we designed were blunt-ended, we synthesized additional dsiRNAs carrying dTdT overhangs at the 3′-end of both strands (Fig. 2A) and tested their gene silencing activities. The gene silencing efficiency of dsiRNAs with 2 nt 3′-overhang was similar to that of blunt-ended dsiRNAs (Fig. 2B). However, the 3′-overhang structure could be important for reducing the antiviral responses triggered by long dsRNAs (See below).

DsiRNAs execute target gene silencing via the RNAi pathway utilized by conventional siRNAs

To test whether these dsiRNAs trigger gene silencing through the RNA interference pathway utilized by conventional 19 bp siRNAs, we analyzed the cleavage sites of each target mRNA cleaved by dsiRNAs using 5′-RACE (Rapid amplification of cDNA ends) assay (Soutschek et al., 2004; Yekta et al., 2004). All dsiRNAs produced the 5′-RACE PCR products with sizes similar to that produced by conventional 19 bp siRNAs, suggesting that the cleavage sites within the target mRNA is similar (Figs. 3A and 3B). For the detailed characterization of the cleavage sites within the target mRNAs, each 5′-RACE products were cloned and sequenced. All dsiRNA structural variants cleaved the target mRNAs at the sites corresponding to either the position 9 or 10 nt from the 5′-end of antisense strand of each siRNA unit, which was similar to the conventional 19 bp siRNAs (Figs. 3C and 3D). These results suggest that the spacing between the 5′-end of the guide strand and the cleavage site is identical between dsiRNAs and 19 bp siRNAs.

Next, to test whether the dsiRNAs silence genes via the same RNAi pathway used by conventional siRNAs, we knocked-down Argonaute-2 (Ago2) levels using an siRNA-targeting Ago2 (siAgo2) in HeLa cells (Chang et al., 2009). DsiRNAs were then transfected into HeLa cells treated with or without siAgo2, and the gene silencing efficiencies were analyzed. As shown in Fig. S5, Lamin A/C gene silencing activities of siRNA and dsiRNAs were reduced following Ago2 knockdown. This result suggests that dsiRNAs trigger gene silencing in an Ago2-dependent manner, like conventional siRNAs. Altogether, we conclude that dsiRNA structural variants execute gene silencing through the same RNAi pathway utilized by the conventional siRNAs.

Reduced sense-strand mediated off-target gene silencing by dsiRNAs

One undesired effect triggered by siRNAs is the sense strand-mediated off-target gene silencing (Clark et al., 2008), a phenomenon in which the sense strand of siRNA is incorporated into RISC, that knocks-down the unintended target genes. Because, in our dsiRNA structures, 5′-end of the sense strand are headed toward inside of the duplex structure, we expected that the gene silencing activity of sense strand should be compromised (Chang et al., 2007). To confirm this, we designed vector constructs that encode luciferase mRNA bearing sense-target or antisense-target sequence of siTIG3 at the 3′-untranslated region. These constructs were then transfected into HeLa cells along with siTIG3 alone, mixture of siLamin and siTIG3, and dsiLamin(19)-TIG3(19), respectively, and the level of luciferase activity was measured. Regardless of the thermodynamically unstable design of the 5′-end of antisense strand for preferential incorporation of antisense strand into the active RISC (Schwarz et al., 2003), siTIG3 showed strong sense strand-mediated gene silencing activities when either transfected alone or co-transfected with siLamin (Fig. S6). In contrast, dsiLamin(19)-TIG3(19) showed reduced silencing activity for siTIG3 sense-target while it efficiently silenced siTIG3 antisense-target (Figs. S6A and S6B). This result demonstrates that our dsiRNA structures could be more specific gene silencers than conventional 19 bp siRNAs, by reducing the sense strand-mediated off-target silencing of siRNA

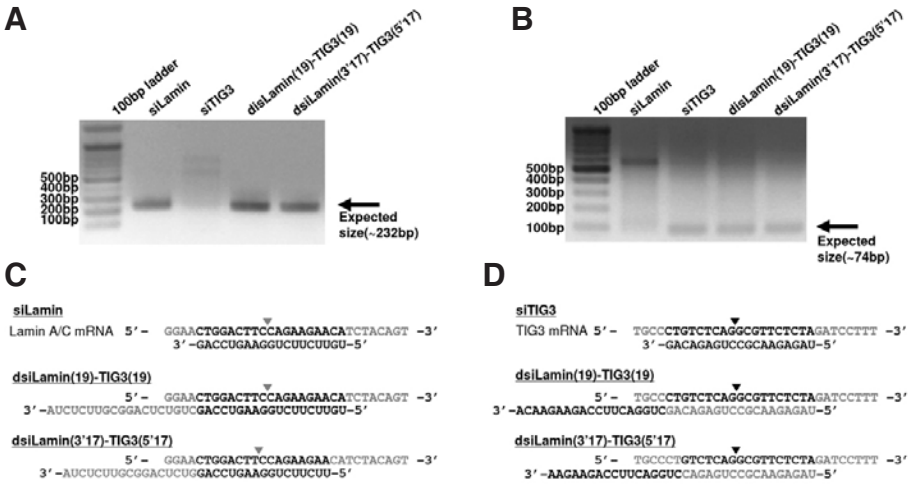

Fig. 3. DsiRNAs work via RNAi mechanism used by conventional siRNAs. (A) PCR products of Lamin A/C specific 5′-RACE assay from dsiRNA-transfected cells. (B) PCR products of TIG3-specific 5′-RACE assay from dsiRNA-transfected cells. (C) Cleavage sites of Lamin A/C mRNA were analyzed by 5′-RACE assay and sequencing. Antisense sequences of siLamin A/C part from siRNA or dsiRNAs are shown as the bottom strand, and cleavage sites are marked with arrow heads. (D) Cleavage sites of TIG3 mRNA were analyzed by 5′-RACE assay and sequencing. Antisense sequences of siTIG3 part from siRNA or dsiRNAs are shown as the bottom strand, and cleavage sites are marked with arrow heads.

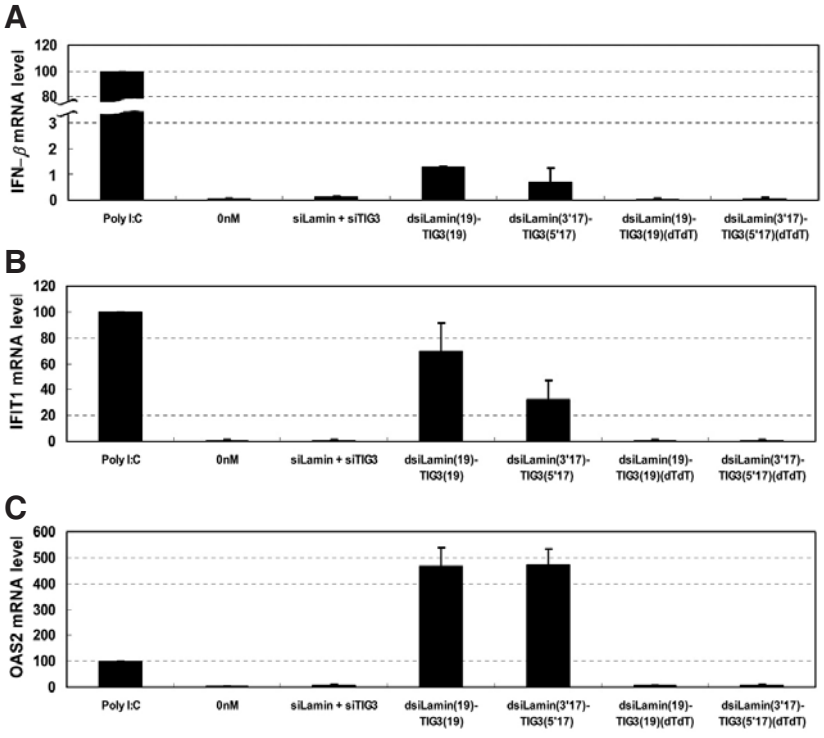

Fig. 4. Antiviral responses triggered by dsi RNA structural variants. (A) *IFN-β* mRNA induction levels of T98G cells transfected with dsiRNAs. See the legend of Fig. 1B for details. (B) *IFIT1* mRNA induction levels of T98G cells transfected with dsiRNAs. (C) *OAS2* mRNA induction levels of T98G cells transfected with dsiRNAs.

units. We would like to mention that, because of poor gene silencing activity of siLamin sense-strand, we did not test the siLamin sense-target activity for our dsiRNAs (data not shown).

Antiviral innate immune responses of dsiRNAs

Although our dsiRNA structures could trigger efficient gene silencing, they might be able to trigger antiviral responses on account of their longer length, as compared with 19 bp siRNA duplex (Elbashir et al., 2001b; Manche et al., 1992). We therefore assessed the antiviral responses of dsiRNAs by measuring the induction levels of *IFN-β*, *IFIT1*, and *OAS2* mRNAs from dsiRNA-treated cells. As a positive control for antiviral gene induction, we used poly(I:C)-transfected cells (Kim et al., 2007) (Fig. 4) . In HeLa cells, none of the dsiRNA structures evoked significant induction in IFN-*β*, *IFIT1*, and *OAS2* mRNA level (data not shown). Because HeLa cells were known to be rather insensitive to siRNA-mediated innate immune responses (Reynolds et al., 2006), we also tested the non-specific immune response in T98G cells, which were reported to be immune-sensitive to the siRNA treatment (Marques et al., 2006). Indeed, in T98G cells, blunt-ended, 38 and 34 bp-long dsiRNAs triggered significant level of IFN-*β*, *IFIT1*, and *OAS2* induction (Fig. 4).

Marques et al. (2006) reported that the presence of 2 nt 3′-overhangs could alleviate non-specific immune responses triggered by siRNA duplexes up to 27 bp long. To test whether this could be also applied to the RNA duplexes longer than 30 bp, T98G cells were transfected with 38 and 34 bp-long dsiRNAs bearing 3′-dTdT overhangs and antiviral gene induction levels

were measured. We found that the 38 and 34 bp-long dsiRNAs with 3′-overhangs did not induce any of the antiviral genes tested (Fig. 4). These results emphasize that, contrary to the previous report (Elbashir et al., 2001b), one could use RNA duplexes as long as 38 bp to trigger gene specific RNAi without non-specific immune responses, even in immune-sensitive cell lines such as T98G.

DISSCUSSION

In this study, we constructed a variety of dsiRNA structures that could efficiently silence two target genes simultaneously. All dsiRNAs were designed with their 5′-end of antisense strand headed toward outside of RNA core structures, as this combination provided good gene silencing activities for both siRNA units used. This connection strategy seems to work on account of the open seed region at the at 5′-end of antisense strand of an siRNA. A key observation from our previous study, which shows that an attachment of a heterologous duplex to the 5′-end of an antisense strand results in reduced gene silencing (Chang et al., 2007), also confirms the importance of an undisturbed and accessible seed region. We constructed dsiRNAs based on the directionality rule, and all four dsiRNAs synthesized successfully executed gene silencing for both target genes. This suggests that this combination strategy could be highly applicable to a wide range of siRNA combination to construct dsiRNA structure. Thus, our directionality rule could be helpful guides in constructing dsiRNA structures with high success rates.

Contrary to the current belief, while the duplex length of the dsiRNA structures we made was longer than 30 bp, they did not induce significant interferon responses. A similar result has been recently reported by others where specific single gene silencing was achieved using 40 bp-long siRNA duplex (Vickers et al., 2007). Although significant antiviral gene induction was observed by 38 and 34 bp-long siRNAs in an immune-competent cell line such as T98G (Marques et al., 2006), these dsiRNAs did not trigger non-specific global mRNA degradation, which would be observed upon extensive antiviral interferon responses. More importantly, we could successfully control the antiviral gene induction by 38 and 34 bp-long dsiRNAs by adding 2 nt overhangs on their 3′-ends, as previously reported (Marques et al., 2006). Combined together, these results demonstrate that dsRNA as long as 38 bp can result in specific target gene silencing without non-specific antiviral responses. Therefore, the original hypothesis that, RNA duplexes longer than 30 bp could not trigger RNAi due to the induction of potent antiviral responses, needs to be revised. This will give researchers more room to design diverse multifunctional siRNA structures.

The successful construction of these novel, long dsiRNA structures supports the idea that gene silencing siRNA structures are more flexible than originally proposed (Chang et al., 2009), and expands the structural diversity repertoire of siRNAs suitable for multiple gene silencing. We believe that these novel dsiRNA structures have the potential to become a useful strategy for siRNA-based antiviral or anticancer therapeutics development, which can be benefited by simultaneous silencing of two target genes.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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REFERENCES

Birmingham, A., Anderson, E.M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., Baskerville, S., Maksimova, E., Robinson, K., Karpilow, J., et al. (2006). 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat. Methods *3*, 199-204.

Boden, D., Pusch, O., Lee, F., Tucker, L., and Ramratnam, B. (2003). Human immunodeficiency virus type 1 escape from RNA interference. J. Virol. *77*, 11531-11535.

Caplen, N.J., Fleenor, J., Fire, A., and Morgan, R.A. (2000). dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference. Gene *252*, 95-105.

Chang, C.I., Hong, S.W., Kim, S., and Lee, D.K. (2007). A structure-activity relationship study of siRNAs with structural variations. Biochem. Biophys. Res. Commun. *359*, 997-1003.

Chang, C.I., Yoo, J.W., Hong, S.W., Lee, S.E., Kang, H.S., Sun, X., Rogoff, H.A., Ban, C., Kim, S., Li, C.J., et al. (2009). Asymmetric shorter-duplex siRNA structures trigger efficient gene silencing with reduced nonspecific effects. Mol. Ther. *17*, 725-732.

Clark, P.R., Pober, J.S., and Kluger, M.S. (2008). Knockdown of TNFR1 by the sense strand of an ICAM-1 siRNA: dissection of an off-target effect. Nucleic Acids Res. *36*, 1081-1097.

Das, A.T., Brummelkamp, T.R., Westerhout, E.M., Vink, M., Madiredjo, M., Bernards, R., and Berkhout, B. (2004). Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J. Virol. *78*, 2601-2605.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001a). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. *15*, 188-200.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001b). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494-498.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001c). Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J. *20*, 6877-6888.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

Grimm, D., and Kay, M.A. (2007). Combinatorial RNAi: a winning strategy for the race against evolving targets? Mol. Ther. *15*, 878-888.

Hossbach, M., Gruber, J., Osborn, M., Weber, K., and Tuschl, T. (2006). Gene silencing with siRNA duplexes composed of target-mRNA-complementary and partially palindromic or partially complementary single-stranded siRNAs. RNA Biol. *3*, 82-89.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P.S. (2003). Expression profiling reveals off-target gene regulation by RNAi. Nat. Biotechnol. *21*, 635-637.

Jackson, A.L., Burchard, J., Leake, D., Reynolds, A., Schelter, J., Guo, J., Johnson, J. M., Lim, L., Karpilow, J., Nichols, K., et al. (2006). Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA *12*, 1197-1205.

Jacque, J.M., Triques, K., and Stevenson, M. (2002). Modulation of HIV-1 replication by RNA interference. Nature *418*, 435-438.

Kennerdell, J.R., and Carthew, R.W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell *95*, 1017-1026.

Khaled, A., Guo, S., Li, F., and Guo, P. (2005). Controllable self-assembly of nanoparticles for specific delivery of multiple therapeutic molecules to cancer cells using RNA nanotechnology. Nano Lett. *5*, 1797-1808.

Khvorova, A., Reynolds, A., and Jayasena, S.D. (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell *115*, 209-216.

Kim, D.H., Behlke, M.A., Rose, S.D., Chang, M.S., Choi, S., and Rossi, J.J. (2005). Synthetic dsRNA Dicer substrates enhance

RNAi potency and efficacy. Nat. Biotechnol. *23*, 222-226.
Kim, J.Y., Choung, S., Lee, E.J., Kim, Y.J., and Choi, Y.C. (2007). Immune activation by siRNA/liposome complexes in mice is sequence- independent: lack of a role for Toll-like receptor 3 signaling. Mol. Cells *24*, 247-254.
Konstantinova, P., de Vries, W., Haasnoot, J., ter Brake, O., de Haan, P., and Berkhout, B. (2006). Inhibition of human immunodeficiency virus type 1 by RNA interference using long-hairpin RNA. Gene Ther. *13*, 1403-1413.
Liu, Y.P., Haasnoot, J., and Berkhout, B. (2007). Design of extended short hairpin RNAs for HIV-1 inhibition. Nucleic Acids Res. *35*, 5683-5693.
Manche, L., Green, S.R., Schmedt, C., and Mathews, M.B. (1992). Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol. Cell. Biol. *12*, 5238-5248.
Marques, J.T., Devosse, T., Wang, D., Zamanian-Daryoush, M., Serbinowski, P., Hartmann, R., Fujita, T., Behlke, M.A., and Williams, B.R. (2006). A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. Nat. Biotechnol. *24*, 559-565.
Menendez, J.A., Vellon, L., Mehmi, I., Oza, B.P., Ropero, S., Colomer, R., and Lupu, R. (2004). Inhibition of fatty acid synthase (FAS) suppresses HER2/neu (erbB-2) oncogene overexpression in cancer cells. Proc. Natl. Acad. Sci. USA *101*, 10715-10720.
Nishitsuji, H., Ikeda, T., Miyoshi, H., Ohashi, T., Kannagi, M., and Masuda, T. (2004). Expression of small hairpin RNA by lentivirus-based vector confers efficient and stable gene-suppression of HIV-1 on human cells including primary non-dividing cells. Microbes Infect. *6*, 76-85.
Novina, C.D., Murray, M.F., Dykxhoorn, D.M., Beresford, P.J., Riess, J., Lee, S.K., Collman, R.G., Lieberman, J., Shankar, P., and Sharp, P.A. (2002). siRNA-directed inhibition of HIV-1 infection. Nat. Med. *8*, 681-686.
Reynolds, A., Anderson, E.M., Vermeulen, A., Fedorov, Y., Robinson, K., Leake, D., Karpilow, J., Marshall, W.S., and Khvorova, A. (2006). Induction of the interferon response by siRNA is cell type- and duplex length-dependent. RNA *12*, 988-993.
Sano, M., Li, H., Nakanishi, M., and Rossi, J.J. (2008). Expression of long anti-HIV-1 hairpin RNAs for the generation of multiple siRNAs: advantages and limitations. Mol. Ther. *16*, 170-177.
Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P.D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell *115*, 199-208.
Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., et al. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature *432*, 173-178.
Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., and Schreiber, R.D. (1998). How cells respond to interferons. Annu. Rev. Biochem. *67*, 227-264.
ter Brake, O., Konstantinova, P., Ceylan, M., and Berkhout, B. (2006). Silencing of HIV-1 with RNA interference: a multiple shRNA approach. Mol. Ther. *14*, 883-892.
Ui-Tei, K., Zenno, S., Miyata, Y., and Saigo, K. (2000). Sensitive assay of RNA interference in Drosophila and Chinese hamster cultured cells using firefly luciferase gene as target. FEBS Lett. *479*, 79-82.
Vickers, T.A., Lima, W.F., Nichols, J.G., and Crooke, S.T. (2007). Reduced levels of Ago2 expression result in increased siRNA competition in mammalian cells. Nucleic Acids Res. *35*, 6598-6610.
Watanabe, T., Sudoh, M., Miyagishi, M., Akashi, H., Arai, M., Inoue, K., Taira, K., Yoshiba, M., and Kohara, M. (2006). Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. Gene Ther. *13*, 883-892.
Westerhout, E.M., Ooms, M., Vink, M., Das, A.T., and Berkhout, B. (2005). HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. Nucleic Acids Res. *33*, 796-804.
Yekta, S., Shih, I.H., and Bartel, D.P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. Science *304*, 594-596.
Yoo, J.W., Kim, S., and Lee, D.K. (2007). Competition potency of siRNA is specified by the 5′-half sequence of the guide strand. Biochem. Biophys. Res. Commun. *367*, 78-83.
Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell *101*, 25-33.