Long Double-Stranded Multiplex siRNAs for Dual Genes Silencing

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Simultaneous suppression of multiple oncogenes is an attractive strategy to treat cancers. Herein we present a series of long double-stranded multiplex small interfering RNAs (multi-siRNAs) that is suitable for dual genes silencing through a sequence-specific RNA interference process without inducing significant immune responses. A gap feature structurally designed in either of the nucleotide strands of the multi-siRNAs was proved to be essential toward silencing target genes and avoiding immune responses. Furthermore, the silencing effect of multi-siRNAs against *SURVIVIN* and *BCL-2* genes was shown to be effective and resulted in up-regulation of caspase-3 related apoptosis and, in turn, inhibition of bladder cancer cell proliferation. Our observation suggested that the rationally designed multi-siRNAs would have great potential for therapeutic siRNA design.

Introduction

RNA INTERFERENCE (RNAi) is a gene silencing mechanism induced by double-stranded RNA (dsRNA). Naturally, when long dsRNA is introduced into cells, Dicer, a cytoplasmic RNase 3 enzyme, cleaves it into 21- to 23-bp dsRNA with 2-nt 3' overhangs, which is termed small interfering RNA (siRNA) (Zamore et al., 2000). RNA-induced silencing complex (RISC) loads the antisense strand of siRNA then recognizes and cleaves target messenger RNAs (mRNAs) with complementary sequences, which results in effective gene silencing (Elbashir et al., 2001).

Because of its efficient and specific silencing of target gene expression, siRNA has recently been experimentally used to treat cancers. In general, inhibition of a single oncogenic gene is insufficient to kill tumor cells because oncogenesis is associated with multiple genes involved in multiple pathways (Menendez et al., 2004). Therefore, use of multi-targeting siRNAs could be an advantageous strategy, by which expression of multiple genes can be inhibited simultaneously, and thus this may lead to more effective anticancer therapies than what single-targeting siRNA could achieve. In addition to multi-cassette short hairpin RNAs (shRNAs) (Watanabe et al., 2006) and long hairpin RNAs (Sano et al., 2008), studies on the use of synthetic multi-targeting siRNAs have also been reported. Hossbach et al. (Hossbach et al., 2006) reported a dual-targeting siRNA that was composed of 2 fully target-complementary guide strands with the strands partially complementary to each other. An algorithm was further developed to predict dual-targeting siRNAs with complete complementary strands that acted as

active guide strands separately targeting different mRNAs (Tiemann et al., 2010). However, both methods suffered from limitation of the algorithm used, and the designed siRNAs may not be the most effective sequences. A recent study reported that long synthetic siRNA duplexes (38 bp) simultaneously knocked down expression of 2 genes without triggering an interferon response (Chang et al., 2009). This, however, contradicted the results from a previous study, in that dsRNAs longer than 30 bp could not execute specific gene silencing due to an induction of interferon response (Elbashir et al., 2001).

In the present study, we designed a group of new and long double-stranded multiplex siRNA (multi-siRNA) structures, featured with a gap in either sense strand or antisense strand. Results proved that the uniquely designed multi-siRNAs were capable of silencing both *SURVIVIN* and B-cell lymphoma 2 (*BCL-2*) genes effectively in an Argonaute-2 (AGO2)-dependent manner without interferon responses, thus significantly suppressed proliferation of human bladder cancer T24 cells.

Materials and Methods

Sequence design of single-targeting siRNAs and multiplex-siRNAs

Individual siRNAs targeting human *SURVIVIN* mRNA (si-S1 ~ si-S3) were selected from a full-site siRNA library of *SURVIVIN* constructed by Biomics Biotechnologies Co. Ltd. Individual siRNAs targeting human *BCL-2* (si-B1 and si-B2) were designed using Thermo siDESIGN Center and Invitrogen BLOCK-iT RNAi Designer online tools. After screening the most effective single-target siRNA sequences, multi-target

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siRNAs were designed using the sequences listed in Table 1 accordingly. To enhance stability of the multi-target siRNAs, 2'-OMe modification was incorporated in both of the strands on every second nucleotide (Czaderna et al., 2003). siRNA targeting luciferase gene (si-GL3) was used as a negative control. All siRNAs in the study were chemically synthesized by Biomics Biotechnologies.

Cell culture and siRNA transfection

T24 cells were conserved in Biomics and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C under 5% CO₂ humidified atmosphere. Cells grown to a confluence of 30%–40% were transfected with siRNAs by Lipofectamine 2000 according to manufacturer's guidelines. Ten nanomoles of siRNA was used in each case unless stated otherwise.

Quantitative real-time reverse transcription polymerase chain reaction

A RISO™ RNA isolation reagent (Biomics Biotech) was used to extract total RNA from cell lysates. The complementary DNA was synthesized by reverse transcription and expression levels of human *SURVIVIN* and *BCL-2* mRNA were determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) using EzOmics™ one-step qPCR kit (Biomics Biotech) according to manufacturer's instructions. Specific primers used for each gene were as follows:

SURVIVIN forward: 5'-AGGTCATCTCGGCTGTTCCCTG-3'
SURVIVIN reverse: 5'-TCATCCTCACTGCGGCTGTC-3'
BCL-2 forward: 5'-GGCTGGGATGCCTTTGTG-3'
BCL-2 reverse: 5'-GCCAGGAGAAATCAAACAGAGG-3'
OAS1 forward: 5'-GTGAGCTCCTGGATTCTGCT-3'
OAS1 reverse: 5'-TGTTCATGTAACCATATTCTGA-3'
IFIT1 forward: 5'-AAAAGCCCACATTTGAGGTG-3'

TABLE 1. SMALL INTERFERING RNA SEQUENCES AND MODIFICATIONS

siRNA	Sequence
si-S3	5' GACUUGGCCAGUGUUUCU 3' 3' CUGAACCGGGUCACAAAGA 5'
si-B1	5' GGAUGACUGAGUACCUGAA 3' 3' CCUACUGACUAGGACUU 5'
Sub-1	5' GACUUGGCCAGUGUUUCUACUGGAUGA CUGAGUACCGUAA 3' 3' CUGAACCGGGUCACAAAGA CCUACUGAC UCAUGGACUU 5'
Sub-2	5' GACUUGGCCAGUGUUUCU GGAUGACU GAGUACCGUAA 3' 3' CUGAACCGGGUCACAAAGAUGACCUACU GACUACUGGACUU 5'
Sub-3	5' GACUUGGCCAGUGUUUCUACUGGAUGA CUGAGUACCGUAA 3' 3' CUGAACCGGGUCACAAAGAUGACCUACU GACUACUGGACUU 5'
si-GL3	5' CUUACGCUGAGUACUUCGAdTdT 3' 3' dTdTGAUAGCGACUCAUGAAGCU 5'

2'-O-methyl ribonucleotide modifications are underlined.
 siRNA, small interfering RNA.

IFIT1 reverse: 5'-GAAATTCCTGAAACCGACCA-3'
AGO2 forward: 5'-CGACTGCTAGGTGGCGCC-3'
AGO2 reverse: 5'-TCCCAGACGCCCTGGACAGG-3'
GAPDH forward: 5'-GAAGGTGAAGGTCCGAGTC-3'
GAPDH reverse: 5'-GAAGATGGTGATGGGATTTC-3'

Samples were tested in triplicate and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression was used as an internal control.

Western blotting

Cells were seeded in 6-well plates respectively. When confluence of the cells reached 50%, transfection was conducted with siRNA and lipofectamine 2000 as described above. After further incubation for 48 hours, the cells were lysed using a mammalian cell total protein lysis buffer (Sangon Biotech). Fifty micrograms of total proteins were separated in 12% sodium dodecyl sulfate acrylamide gels and electroblotted onto an immobilon polyvinylidene difluoride membrane (Millipore, Billerica). The membranes were blocked for 2 hours in phosphate buffered saline with Tween-20 (PBST) containing 5% skim milk powder and incubated overnight at 4°C with a 1:100 dilution of mouse monoclonal primary antibody to human *SURVIVIN* (Santa Cruz Biotech) or a 1/200 dilution of mouse monoclonal antibody to human *BCL-2* (Santa Cruz Biotech) and human *GAPDH* (Santa Cruz Biotech), respectively. The membranes were then washed in PBST and incubated with a 1/1000 dilution of goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) at room temperature for 2 hours. Upon incubation with an electrochemiluminescence (ECL) chemiluminescence reagent (Beyotime Institute of Biotechnology), the membranes were exposed to films (Kodak).

Interferon responses assay

Transfection and qRT-PCR assays were performed as described above. Expression of 2'-5'-oligoadenylate synthetase 1 (*OAS1*) and interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*) mRNAs were measured. Expression of *GAPDH* was used as an internal control for normalization of qRT-PCR results.

Cell proliferation assay

Cells were seeded in 96-well plates at 2,000 cells per well 24 hours prior to transfection, and the transfection was conducted as described above. At the scheduled time points, cell proliferation capacity was measured in triplicate using a cell counting kit according to manufacturer's specifications (Beyotime Institute of Biotechnology).

Apoptosis analysis

To measure early and late apoptotic cells, the cell samples were stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide using an Annexin V-FITC Apoptosis Detection kit (KeyGen Biotech) and analyzed by FACSscan flow cytometry (BD Biosciences) at 48 hours after siRNA transfection.

Caspase-3 activity assay

Total proteins were extracted from transfected cells using a mammalian cell total protein lysis buffer (Sangon Biotech) as

FIG. 1. Selection of effective small interfering (siRNAs) against *SURVIVIN* and B-cell lymphoma 2 (*BCL-2*) genes. siRNAs targeting human *SURVIVIN* gene are named si-S1, si-S2, and si-S3. si-GL3 is a siRNA silencing luciferase gene and served as a negative control. Quantitative real-time polymerase chain reaction (q-RT-PCR) was performed for siRNAs against either *SURVIVIN* mRNA (**A**) or *BCL-2* mRNA (**B**) 48 hours after transfection. Values were averaged from 3 independent experiments. * indicates significant difference compared with values for si-GL3 treated cells ($P < 0.05$).

described above. Protein concentration of the whole cell extract was measured by Bradford assay. Activity of caspase-3 was determined using a caspase-3 activity kit (Beyotime Institute of Biotechnology) according to manufacturer's instructions. One unit of caspase-3 activity is defined as the amount of enzyme that cleaves 1.0 nmol of colorimetric substrate Ac-DEVD-pNA at a saturated substrate concentration at 37° in 1 hour. Relative caspase-3 activities were expressed as folds of up-regulation compared to those of si-GL3 transfected cells.

Statistical analysis

Data were expressed as means $\pm$ standard deviation. Difference between any 2 groups was determined by one-way analysis of variance and is considered to be significant statistically when P value is < 0.05 .

Results

Selection of effective siRNA targeting human *SURVIVIN* or *BCL-2*

Designed siRNAs targeting human *SURVIVIN* gene (si-S1 ~ si-S3) and human *BCL-2* gene (si-B1 and si-B2) were first screened for their effectiveness, respectively. As shown in Fig. 1, si-S3 transfection resulted in a significant decrease in expression of *SURVIVIN* mRNA, at 79%, compared with that in the negative control, and si-B1 was found to be the most effective siRNA targeting *BCL-2* gene with 84% gene knockdown. The results were further confirmed by western blotting analysis (Fig. 2), in

which si-S3 and si-B1 significantly suppressed the expression of *SURVIVIN* and *BCL-2* proteins respectively.

Effects of multi-siRNAs on *BCL-2* and *SURVIVIN* gene expression

Three unique types of double stranded multi-siRNA structures were designed and synthesized, as shown in Table 1, using the sequences of si-S3 and si-B1 in combination, which were named Sub-1, Sub-2, and Sub-3, respectively. To evaluate their gene silencing efficiency, Sub-1, Sub-2, and Sub-3 were transfected, respectively, into T24 cells by lipofectamine 2000. Expression levels of mRNA and protein from *SURVIVIN* and *BCL-2* genes were determined by qRT-PCR and western blotting assays, respectively. For the cells treated with Sub-1, simultaneous down-regulations of *SURVIVIN* mRNA to about 66% and *BCL-2* mRNA to 83% were achieved, respectively. In comparison, Sub-2 showed somewhat similar down-regulations of *SURVIVIN* (71%) and *BCL-2* (81%) at mRNA levels (Fig. 3). Western blotting assays further demonstrated that both Sub-1 and Sub-2 inhibited the expression of *SURVIVIN* and *BCL-2* proteins more effectively than Sub-3 (Fig. 4). In light of these results, further studies were focused on the use of the more effective multi-siRNAs, Sub-1, and Sub-2.

No significant interferon responses induced by multi-siRNAs

It was previously known that dsRNA longer than 30 bp (Elbashir et al., 2001) could induce interferon (IFN) responses

FIG. 2. Suppression in protein levels of *SURVIVIN* or *BCL-2* gene by siRNAs in T24 cells. (**A**) Suppression of *SURVIVIN* by *SURVIVIN*-specific siRNAs treatment. (**B**) Suppression of *BCL-2* by *BCL-2*-specific siRNAs treatment. si-S1, si-S2, si-S3, si-B1, si-B2, and si-GL3 siRNAs are defined as in Fig. 1.

FIG. 3. Gene-silencing activities of multi-siRNAs against *SURVIVIN* and *BCL-2* genes. siRNAs were transfected into T24 cells by lipofectamine 2000. Twenty-four hours after transfection, *SURVIVIN* (A) or *BCL-2* (B) mRNA levels were quantified by q-RT-PCR. Sub-1 and Sub-2: long double-stranded multi-siRNAs targeting *SURVIVIN* and *BCL-2* genes with a gap in the antisense strand or the sense strand, respectively. Sub-3: the long double-stranded siRNA targeting *SURVIVIN* and *BCL-2* genes with intact sense and antisense strands. si-S3 (targeting *SURVIVIN*) and si-B1 (targeting *BCL-2*) served as individual siRNA controls. si-GL3 is a siRNA silencing luciferase gene and served as a negative control. All data represent means $\pm$ standard deviation (SD) of 3 independent experiments. * indicates significant difference in levels compared with si-GL3 treated cells ($P < 0.05$).

and thus cause non-specific apoptosis (false gene knock-down). To assess if a potential influence of IFN response happened on the multi-siRNAs (Sub-1 and Sub-2) mediated gene silencing effects, the RNA-transfected cells were analyzed for mRNA expressions of *OAS1* and *IFIT1* at 48 hours after the transfection, along with the si-S3 and si-B1 (siRNAs <21bp) treated cells as negative controls. As shown in Fig. 5, similar to si-S3 or si-B1 negative controls, the mRNA expressions of *OAS1* and *IFIT1* were not significantly increased in the Sub-1 and Sub-2 transfected cells. In contrast, significant increases in *OAS1* and *IFIT1* mRNA expressions were only observed in the Sub-3 transfected cells, reaching 1.8-fold and 5.4-fold higher than negative control, respectively.

Gene silencing by multi-siRNAs in an AGO2-dependent manner

To study whether the multi-siRNAs silence the genes via the same AGO2-dependent pathway as that of conventional siRNAs, knock-down of AGO2 expression was first implemented by a known effective siRNA (Fig. 6), and then mRNA levels of *SURVIVIN* and *BCL-2* genes were measured, respectively, after further transfection with targeting siRNAs or multi-siRNAs. As shown in Fig. 7, the knock-down of both *SURVIVIN* and *BCL-2* genes by either individual siRNAs (si-S3 and si-B1) or multi-siRNAs (Sub-1 and Sub-2) was effectively blocked after pretreatment of cells with AGO2 gene-specific siRNA. The result supported that the effect of gene silencing achieved by the multi-siRNAs did follow an AGO2-dependent pathway.

Inhibition of cell proliferation by multi-siRNAs

At 48, 72, 96, and 120 hours after transfection, inhibition of cell proliferation was observed in all the targeting siRNA groups compared to the negative control group (Fig. 8). At 48 hours after the treatment with 10 or 20 nM of si-S3, the relative proliferation rates of T24 cells were $80.36\% \pm 5.61\%$ and $57.82\% \pm 11.85\%$, respectively ($P < 0.05$, compared to the si-GL3 control). The same trend was also observed in si-B1, Sub-1, and Sub-2 transfected groups, in which the relative proliferation rates decreased with an increased amount of the siRNAs used. The results indicated that the effect on growth inhibition was siRNA dose-dependent. Furthermore, it was noted that the inhibitory effect of the siRNAs was also time-dependent to a certain extent. For instance, cells transfected with Sub-1 for 48, 72, 96, and 120 hours showed a relative viability in rates, distributed at $31.21\% \pm 2.29\%$, $23.24\% \pm 2.36\%$, $27.30\% \pm 0.96\%$, and $14.55\% \pm 0.40\%$, respectively. In addition, it was also observed that at the higher dose of 20 nM, the multi-siRNAs (Sub-1 and Sub-2) transfection produced greater suppression activity than either single-target siRNAs (si-S3 and si-B1) or the combination of si-S3 and si-B1 (10 nM + 10 nM) treatment ($P < 0.05$) at all the measuring time points.

Enhancement of cell apoptosis by multi-siRNAs

Since *SURVIVIN* and *BCL-2* are known as the anti-apoptosis genes, blocking of *SURVIVIN* or *BCL-2* gene is expected to cause an increase in apoptosis (Kunze et al., 2008; Montazeri et al, 2011). Rates of apoptosis were measured in T24 cells after treatment with siRNAs by flow cytometry assays.

FIG. 4. Suppression of *SURVIVIN* (A) and *BCL-2* (B) expression by multi-siRNAs. si-S3, si-B1, Sub-1, Sub-2, Sub-3, and si-GL3 siRNAs are defined as in Fig. 3.

FIG. 5. Interferon responses triggered by siRNAs. **(A)** Oligoadenylate synthetase 1 (*OAS1*) mRNA levels in T24 cells induced by siRNA transfection. **(B)** Tetratricopeptide repeats 1 (*IFIT1*) mRNA levels in T24 cells transfected with siRNAs. si-S3, si-B1, si-GL3, Sub-1, Sub-2, and Sub-3 siRNAs are defined as in Fig. 3. Data were averaged from 3 independent experiments. * $P < 0.05$ compared with si-GL3 cells.

As shown in Fig. 9, individual si-S3 or si-B1 treatment produced an augmentation of apoptosis to 20.96% and 18.45%, respectively, compared with the negative control, which displayed an apoptosis rate of 6.35%. An increase in apoptosis rate induced by the multi-siRNAs was also observed. The apoptosis in Sub-1 and Sub-2 treatments reached to 18.63% and 23.63%, respectively.

Up-regulation of caspase-3 activity by multi-siRNAs

It is known that apoptosis involves a cascade of proteolytic reactions affected mainly by the caspase family, and caspase-3 is thought to be the major executor of apoptosis (Porter and Janicke, 1999). Therefore, the levels of the caspase-3 activity were analyzed after siRNA treatments, as shown in Fig. 10. Based on apoptotic caspase-3 activity, the si-S3 and si-B1 transfection groups reached 1.57-fold and 1.77-fold increases in apoptosis respectively, compared with the si-GL3 transfection group ($P < 0.05$); whereas, Sub-1 and Sub-2 treatment groups demonstrated much greater increases in caspase-3 activities (3.70-fold and 3.64-fold higher than si-GL3 control, respectively, $P < 0.05$).

FIG. 6. Gene-silencing activity of si-Argonaute-2 (*AGO2*) against *AGO2* gene. *AGO2* mRNA level was quantified by q-RT-PCR after si-Ago2 transfection. All data represent means $\pm$ SD of 3 independent experiments. * $P < 0.05$ compared with si-GL3 treated cells.

Discussion

In the present study, we designed 2 new multi-siRNA structures composed of an intact long strand (at least 30 nucleotides in length) and 2 complementary shorter strands. There is a gap between the 2 shorter strands, which results in the segmentation of the complementary strand. A variety of multi-siRNAs targeting human *SURVIVIN* and human *BCL-2* genes was synthesized, and the results proved that Sub-1 and Sub-2 with a gap in either of the strands could effectively silence 2 target genes simultaneously with high potency compared with the corresponding single-target siRNAs. In contrast, gene knock-down efficacy of long double-stranded siRNA (Sub-3) was poor (Figs. 3, 4), although there was no difference in the nucleotide sequences between Sub-3 and Sub-1 (or Sub-2). The results indicated that the gap in either anti-sense or sense strand is essential to the silencing activities of the multi-siRNAs, and knock-down potency of the multi-siRNAs depends on the specific structures to a certain extent. It is well known that long dsRNA in cells will serve as substrates to be digested by a dsRNA-specific endonuclease, Dicer, into siRNA duplexes as functional effectors to cleave target mRNAs. Therefore, the gap between the two complementary strands may provide recognizable sites and/or facilitate the process of Dicer, thus the resultant two siRNAs would then silence their targets, respectively. However, an exact mechanism remains unclear and requires further studies.

A preliminary study on the mechanism of specific gene silencing effects induced by the multi-siRNAs was further studied. It has been widely accepted that siRNA-directed RNA interference is mediated by AGO2. As a core component of RISC, AGO2 protein functions as a slicer to cleave target mRNA and offers a binding site for siRNA in RISC assembly (Liu et al., 2004; Rand et al., 2005). Therefore, the function of AGO2 on the multi-siRNAs induced gene silencing was investigated. As shown in Fig. 7, the gene silencing effects of the multi-siRNAs were blocked when expression of the *AGO2* gene was down regulated. Thus it can be concluded that the silencing effect induced by the multi-siRNAs also requires AGO2 as a catalysis and is likely to be AGO2-dependent, and it follows the same pathway as that utilized by conventional siRNAs.

A previous study reported that dsRNA longer than 30 bp could not execute specific gene silencing because of induction of significant interferon responses after being transfected into mammalian cells (Elbashir et al., 2001). *IFIT1* was identified as a gene that was most strongly induced in response to

FIG. 7. Multi-siRNAs trigger gene silencing in an *AGO2*-dependent manner. Relative *SURVIVIN* (A) and *BCL-2* (B) mRNA levels were determined by q-RT-PCR after indicated siRNAs treatment following *AGO2* mRNA knock-down (si-Ago2 transfection). si-S3, si-B1, si-GL3, Sub-1, and Sub-2 siRNAs are defined as in Fig. 3. Values represent the means averaged from 3 independent experiments.

treatment with dsRNA (Geiss et al., 2001). In addition, *OAS1*, a classical interferon target gene, could be induced by both type 1 and type 2 IFNs (Eskildsen et al., 2003). Therefore, *OAS1* and *IFIT1* were used in this study as sensitive biomarkers to evaluate immune responses resulted from dsRNA. The levels of *OAS1* and *IFIT1* mRNAs from the multi-siRNAs treated T24 cells were measured against those from a conventional siRNA, which was used as a control. The results indicated that the multi-siRNAs with a gap in either of the strands did not evoke significant interferon responses, nor did single-target siRNAs. However, long double-stranded RNA (Sub-3) transfection induced significant increase in the *IFIT1* and *OAS1* mRNA levels, although Sub-3 is also selectively modified by 2'OMe nucleotides. Therefore, a conclusion could be drawn that the lack of an immune response from the multi-siRNAs was due to the specific structures to some extent. These findings demonstrated that *in vitro* gene silencing activities of the multi-siRNAs against respective target genes were derived from sequence-specific RNA interference rather than an IFN response.

A presently accepted notion is that anticancer agents are effective to lead to death of tumor cells only when they induce apoptosis. However, apoptosis is frequently dysregulated in tumor cells, by which cancer cells become resistant to anticancer drug treatments. Overexpression of anti-apoptotic genes, such as *BCL-2* and *SURVIVIN*, has been shown to play a critical role in deficiency of apoptosis (Kasibhatla and Tseng, 2003; Varfolomeev and Vucic, 2011). In the study, multi-siRNAs (Sub-1 and Sub-2) targeting *SURVIVIN* and *BCL-2* genes simultaneously was designed in an attempt to mitigate the

apoptosis deficiency on bladder cancer T24 cells. The data revealed that suppression of either *SURVIVIN* or *BCL-2* gene individually resulted in a small decrease in cell growth and a modest increase in spontaneous cell apoptosis. In contrast, when simultaneous suppression of both *SURVIVIN* and *BCL-2* genes was achieved by Sub-1 or Sub-2, the proliferation of T24 cells was dramatically inhibited and the apoptosis was enhanced by a significant activation of caspase-3. Therefore, the effects of Sub-1 and Sub-2 against cancer cell growth could be related to apoptosis induction.

It can also be noted that the multi-siRNAs with a gap in either of the strands were demonstrated to be more effective on inhibition of cancer cell growth than individual siRNAs used either alone or as a cocktail in the present experiment. There might be two reasons that could account for the improvement: (1) It was previously reported that co-transfection of siRNAs could result in reduced knock-down activity because of competition between siRNAs (Tanudji et al., 2010); therefore, the activity of some siRNAs within the cocktail may be reduced, which could attenuate the effects of the mixture. (2) The multi-siRNAs were transfected into cells as an intact molecule and were then cleaved by Dicer into 2 separate siRNAs to silence respective targets in the same cell. A recent comparison of the efficacy of Dicer intact substrate RNAs and chemically synthesized non-Dicer substrate siRNAs shows that both triggers are potent in target-gene silencing. Differences could, however, be seen when comparing specificity. Many non-Dicer substrate siRNA molecules are easily to produce an off-target effect, whereas the mixture of Dicer intact substrate RNAs leads to a more specific knockdown

FIG. 8. Relative viability of T24 cells after siRNA treatments. T24 cell proliferation was analyzed by a cell counting kit 8 assay at the indicated time points after (A) 10 nM or (B) 20 nM siRNA transfection. si-S3, si-B1, si-GL3, Sub-1, and Sub-2 siRNAs are defined as in Fig. 3. Cell proliferation following siRNA treatment was normalized to that of si-GL3 treatment. * $P < 0.05$ compared with the negative control siRNA (si-GL3) group. # $P < 0.05$ compared with the mix of si-S3 (10 nM) and si-B1 (10 nM) combination group.

FIG. 9. Induction of apoptosis in T24 cells 48 hours after siRNA treatment. Apoptosis rates were measured by fluorescence activated cell sorting analysis after AnnexinV-propidium iodide (PI) staining. Percentage of apoptotic cells included both early- and late-stage apoptosis (AV+/PI- and AV+/PI+). Increases in apoptosis were calculated relative to si-GL3 control. si-S3, si-B1, si-GL3, Sub-1, and Sub-2 siRNAs are defined as in Fig. 3.

FIG. 10. Activity of caspase-3 increased after siRNA transfection. Activity of caspase-3 was quantified by colorimetric assay and normalized to that of the si-GL3 control group. Values were averaged from three independent experiments. * $P < 0.05$ compared with si-GL3 group; # $P < 0.05$ compared with si-S3 group. si-S3, si-B1, si-GL3, Sub-1, and Sub-2 siRNAs are defined as in Fig. 3.

(Kittler et al., 2007). The efficiency of gene silencing primarily relies on the optimal incorporation of siRNA into RISC, where the Dicer substrate RNAs have a preference for RISC loading as a consequence of the physical transfer of the processed siRNA from Dicer (Amarzguioui et al., 2008). Therefore, it was probably on these reasons that multi-siRNAs used in the present experiment showed more effective activity than individual siRNA mixture.

In summary, it was demonstrated that the multi-siRNAs could be potentially utilized for attaining silencing of multiple genes without eliciting an immune response, and the unique design of a gap in either sense strand or anti-sense strand in the multi-siRNAs was proved to be necessary to knock down multiple genes and to avoid immune responses. The results also revealed that the gene knock-down effects resulted from the multi-siRNAs were derived from sequence-specific RNA interference rather than an IFN response. Furthermore, the designed multi-siRNAs targeting both *SURVIVIN* and *BCL-2* genes dramatically inhibited cell growth on bladder cancer T24. Therefore, this new type of multi-siRNAs may have a great potential toward siRNA-based therapeutic applications.

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Author Disclosure Statement

No competing financial interests exist.

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