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A novel siRNA validation system for functional screening and identification of effective RNAi probes in mammalian cells

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Abstract

Small interfering RNAs (siRNAs) have become the most powerful and widely used gene silencing reagents for reverse functional genomics and molecular therapeutics. The key challenge for achieving effective gene silencing in particular for the purpose of the therapeutics is primarily dependent on the effectiveness and specificity of the RNAi targeting sequence. However, only a limited number of siRNAs is capable of inducing highly effective and sequence-specific gene silencing by RNA interference (RNAi) mechanism. In addition, the efficacy of siRNA-induced gene silencing can only be experimentally measured based on inhibition of the target gene expression. Therefore, it is important to establish a fully robust and comparative validating system for determining the efficacy of designed siRNAs. In this study, we have developed a reliable and quantitative reporter-based siRNA validation system that consists of a short synthetic DNA fragment containing an RNAi targeting sequence of interest and two expression vectors for targeting reporter and triggering siRNA expression. The efficacy of the siRNAs is measured by their abilities to inhibit expression of the targeting reporter gene with easily quantified readouts including enhanced green fluorescence protein (EGFP) and firefly luciferase. Using fully analyzed siRNAs against human hepatitis B virus (HBV) surface antigen (HBsAg) and tumor suppressor protein p53, we have demonstrated that this system could effectively and faithfully report the efficacy of the corresponding siRNAs. In addition, we have further applied this system for screening and identification of the highly effective siRNAs that could specifically inhibit expression of mouse matrix metalloproteinase-7 (MMP-7), Epstein–Barr virus (EBV) latent membrane protein 1 (LMP1), and human serine/threonine kinase AKT1. Since only a readily available short synthetic DNA fragment is needed for constructing this novel reporter-based siRNA validation system, this system not only provides a powerful strategy for screening highly effective siRNAs but also implicates in the use of RNAi for studying novel gene function in mammals.

Keywords: siRNA; RNAi; pDual; EGFP; Firefly luciferase; HBsAg; p53; MMP-7; LMP1; AKT1

RNA interference (RNAi) is an evolutionarily conserved mechanism of post-transcriptional gene silencing induced by double-stranded (dsRNA) [1,2]. Primarily, it is involved in the response to exogenous pathogenic and endogenous parasitic nucleic acids [3,4], as well as in the basic cellular functions, such as gene regulation and heterochromatin formation [5–7]. Currently, RNAi has been widely used not only as an extremely powerful approach for reverse functional genomics [8–11], but also as an effectively potent strategy for gene silencing-based therapeutics [12,13]. As compared with other gene silencing reagents, such as antisense oligonucleotides (ODNs), ribozymes, and DNAzymes, siRNAs have apparently become the most powerful and widely used gene silencing reagents for manipulating gene activity in mammalian systems [14]. There are mainly two approaches in generating active siRNAs in mammalian cells by exogenous delivery of synthetic siRNAs [15,16] or short hairpin RNAs (shRNAs) [17], and

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endogenous vector-expressed dsRNAs including shRNAs [18–21] or siRNAs formed by annealing two complementary sense and antisense RNAs [22–24].

Tremendous experimental evidence has already shown that not all of the RNAi targeting sequences selected from a specific gene exhibit the same effectiveness on inhibiting gene expression. Only a limited number of siRNAs is capable of inducing highly effective target gene silencing in a sequence-specific manner [25]. Moreover, the efficacy of siRNAs is dependent on the specificity of the target sequences within a gene and can only be determined experimentally based on the inhibition of the target gene expression. In order to obtain effective siRNAs, it is necessary to design, synthesize, and screen many distinct siRNAs, which are expensive due to the cost of chemical synthesis of RNA oligonucleotides. Although several recent studies have suggested that the secondary structures of mRNA and mRNA binding proteins might interfere with the target site accessibility for RNA-induced silencing complex (RISC), completely dependent on utilizing the rational design strategy for selecting effective siRNAs is not fully programmable [26–29]. In addition, extensively systematic analyses of the siRNA-specific features revealed that siRNA might have sequence specific characteristics associated with its functionality, such as low to medium G/C content (30–50%), high internal stability at the sense strand 5′-terminus, low internal stability at the sense strand 3′-terminus, absence of internal repeats or palindromes, and sense strand base preferences (at positions 1, 3, 10, 13, and 19) [25,30–33]. Thus, these studies indicate that the efficacy of siRNA is not totally secondary structure-dependent, and strongly suggest that the sequence properties of siRNA may play the major and most important role in determining inhibition efficacy.

The key challenge for achieving effective gene silencing in particular for the purpose of the therapeutics is primarily dependent on the effectiveness and specificity of the RNAi targeting sequence. Previously, the effective RNAi probes are identified on the basis of their abilities to inhibit the expression of cognate sequences in an ectopically expressed target gene–reporter fusion chimeric mRNA [34]. The described target gene–reporter-based siRNA validation system totally depends on the availability of cDNA clones, and this may limit the high-throughput application of the method. In addition, sometimes the chimeric mRNA of target gene–reporter fusion construct encodes an impaired fusion protein that exhibits a low reporter activity, which may interfere with the screening and identification of effective RNAi probes. Recently, a reporter-based siRNA validation system has been reported in which the validating system is constructed by fusing a short synthetic DNA fragment containing an RNAi targeting sequence, instead of cDNA, with a reporter gene [35,36]. In this method, however, in order to generate the corresponding triggering siRNA, it is necessary to have either a synthetic siRNA (or shRNA) or another synthetic DNA fragment for constructing siRNA (or shRNA) expression vector. Thus, this system is inefficient and cost-intensive especially for large-scale functional genomic studies.

Not only utilizing the rational design strategy for selecting potentially effective siRNAs but also simultaneously validating the efficacy of designed siRNAs by a simple and reliable standard method could promote large-scale functional genomics in mammalian systems especially for the novel genes. Therefore, it is important to establish a fully robust validating system for determining the efficacy of designed siRNAs. In this study, we have developed a reliable and quantitative reporter-based siRNA validation system for functional screening and identification of effective RNAi probes in mammalian cells. In this system, only a short synthetic DNA fragment is required and used for constructing both the targeting reporter and triggering siRNA expression vectors. By using fully analyzed siRNAs directly against HBsAg or p53 gene expression, we have demonstrated that this reporter-based siRNA validation system could effectively and faithfully report the efficacy of the corresponding siRNAs in a sequence-specific manner. In addition, we have further screened and identified the highly effective siRNAs that could inhibit MMP-7, EBV-LMP1, and AKT1 gene expression by using this system. Since only a readily available short synthetic DNA fragment is needed for constructing both the targeting reporter and triggering siRNA expression vectors, this novel system should not only greatly facilitate large-scale loss-of-function genetic screens in mammalian cells but also provide the basis for an improved approach to screen and identify the most potent siRNA for the purpose of the therapeutics.

Materials and methods

Cell culture. Baby hamster kidney fibroblast BHK, human hepatoma derived cell line Huh7, human cervix epithelioid carcinoma derived cell line HeLa, and human embryonic kidney HEK293 were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated foetal calf serum (FCS; Biological Industries, Kibbutz Beit Haemek, Israel) and 1% antibiotic/antimycotic solution (Gibco-BRL) at 37 °C in a humidified incubator with 5% CO_2. The cell lines were routinely split two to three times a week after treatment with 0.1% trypsin (Biowhittaker Acambrex, Walkersville, MD, USA).

Transfection and luciferase assay. Twenty-four hours before transfection, cells were trypsinized and seeded in 6-well culture plates at 1×10^5 cells per well. The cells were transiently either cotransfected with 0.5 μg of the targeting reporter plasmid or gene expression vector of interest and 1.5 μg of the corresponding siRNA or shRNA expression plasmids, or transfected with 2 μg of the triggering siRNA or shRNA expression plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The EGFP-expressed cells were examined at 48 h later by inverted fluorescent microscopy or were quantified by flow cytometer or Western blot analyses. The luciferase-expressed cells were harvested at 48 h posttransfection and aliquots of the cell lysates containing equal amounts of protein were measured by Dual-luciferase Reporter Assay System (Promega, Madison, WI, USA) as described by the manufacturer. The expression levels of endogenous target genes were determined at 48 h posttransfection by Western blot analysis. The total protein in the cell lysates was determined using the BCA assay (Pierce, Rockford, IL, USA) as recommended by the manufacturer.

Construction of targeting reporter expression vectors. Plasmid vectors were constructed by using standard molecular cloning techniques. The targeting reporter-EGFP plasmids, pEGFP-5UTR, and pEGFP-3UTR (Fig. 2A), containing enhanced green fluorescent protein (*EGFP*) and glutathione *S*-transferase (*GST*) genes, were constructed by inserting the *EGFP* gene from pEGFP-N1 or pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA, USA) and the *GST* gene from pET21a (Novagen, EMD Biosciences, Darmstadt, Germany) into the pCMVη vector (BD Biosciences Clontech) to generate pCMV-EGFP-5UTR or pCMV-EGFP-3UTR and pCMV-GST, respectively, and then the *GST* gene expression cassette from pCMV-GST was cloned into the pCMV-EGFP-5UTR or pCMV-EGFP-3UTR vector. The targeting reporter-firefly luciferase plasmids, pLuc+-5UTR and pLuc+-3UTR (Fig. 2A), containing firefly (*Photinus pyralis*) luciferase (*Fluc*+) and *Renilla* (*Renilla reiformis*) luciferase (*Rluc*+) genes, were constructed by inserting the *Fluc*+ gene from pGL3-Basic (Promega) and the *Rluc*+ gene from pRL-TK (Promega) into the pCMV-EGFP-5UTR or pCMV-EGFP-3UTR and pCMVβ (BD Biosciences Clontech) to generate pCMV-Fluc+-5UTR or pCMV-Fluc+-3UTR and pCMV-Rluc+, respectively, and then the *Rluc*+ gene expression cassette from pCMV-Rluc+ was cloned into the pCMV-Fluc+-5UTR or pCMV-Fluc+-3UTR vector. The targeting reporter-EGFP and firefly luciferase fusion plasmids, pPre-1, 2, and 3 (Fig. 2A), containing *EGFP-Fluc*+ chimeric and *Rluc*+ genes, were constructed by inserting the *Fluc*+ gene from pGL3-Basic into the pEGFP-C1 vector (BD Biosciences Clontech) to generate pEGFP-Fluc+, and then the *EGFP-Fluc*+ expression cassette from pEGFP-Fluc+ was cloned into the pCMVβ vector to produce pCMV-EGFP-Fluc+. To generate the pPre-1, 2, and 3 plasmids, the *Rluc*+ gene expression cassette from pCMV-Rluc+ was further cloned into the pCMV-EGFP-Fluc+ vector. The pPre-1, 2, and 3 vectors contained three different reading frame sequences between EGFP and Fluc+ fused sites, pPre1: 5′-tacaagTCCGGACTCAGATCCGAAGCTTGGAGATCTGAATTCatggaa-3′, pPre2: 5′-tacaagTCCGGACTCAGATCCAAGCTTGGAGATCTGGAATTCatggaa-3′, and pPre3: 5′-tacaagTCCGGACTCAGATCCCGAAGCTTGGGCGAATTCatggaa-3′.

Construction of triggering siRNA expression vector pDual. Oligonucleotides were purchased from commercial suppliers. The RNA polymerase III (Pol III) promoters, human H1 and mouse U6, were PCR-amplified using synthetic oligonucleotides and cloned into the pBluescript II KS (+/−) (Stratagene, La Jolla, CA, USA) to generate pHsH1 (Fig. 2B) and pMmU6. Oligonucleotides used for the amplification of H1 and U6 promoters from human and mouse were: HsH1-S (T7): 5′-TAATACGACTCACTATAGGG-3′ and HsH1-AS: 5′-GAAGATCTGTCTCATACAGAATTATAAAG-3′; MmU6-S: 5′-CCGCTCGAGATCCGACGCCGCCATCTCTAGG-3′ and MmU6-AS: 5′-CCCAAGCTTTTCTCCAAGGGATATTTATAG-3′. To construct the pDual vector (Fig. 2B), the mouse U6 (MmU6) expression cassette from pMmU6 was cloned into the pHsH1 vector.

Construction of targeting reporter and triggering siRNA expression vectors containing a short synthetic DNA fragment of interest. A general strategy for constructing targeting reporter and triggering siRNA (or shRNA) expression vectors involved ligating an annealed oligonucleotide duplex into *Hin*dIII/*Bgl*II restriction site of both expression vectors, simultaneously. Oligonucleotides formed a short synthetic DNA fragment targeting the EGFP, firefly luciferase, HBsAg, p53, mouse MMP-7, EBV-LMP1, and AKT1 genes were purchased from commercial suppliers. The sequences of synthetic DNA oligonucleotides used in this study for constructing siRNA validation system are outlined in Table S1.

Construction of HBV-HBsAg, p53, MMP-7, and EBV-LMP1 expression vectors The p(3A)SAg vector containing HBV-HBsAg gene was kindly provided by Dr. C.-C. Lu (Department of Pathology, National Cheng Kung University, Tainan, Taiwan). The reconstructed HBsAg expression vectors, p(3A)SAg-luc+ and p(3A)SAg-EGFP, contained the firefly luciferase gene from pGL3-Promoter (Promega) and the EGFP gene from pEGFP-N1 (BD Biosciences Clontech) inserted into the p(3A)SAg vector [37]. Construction of the pCMV-p53/EGFP has been described previously [38]. The cDNA encoding mouse MMP-7 or EBV-LMP1 protein was amplified using total RNAs isolated from mouse liver tissue or B95-8 cells with the primers of MMP-7F: 5′-CGGGATCCACCA TGCAGCTCACCCTGTTCTG-3′ and MMP-7R 5′-CCGCTCGAGCGTCACAGCGTGTTCCTCTTTCCATATAAC-3′ or EBV-LMP1F: 5′-CGGAATTCATGGAACACGACCTTGAGAGGG-3′ and EBV-LMP1R: 5′-GGGGTACCAGTCATAGTAGCTTAGCTGAAC-3′ by reverse transcription-polymerase chain reaction (RT-PCR). The amplified PCR fragments were cloned into pcDNA3.1/myc-His vector (Invitrogen) and then confirmed by sequencing. The pCMV-Mat/EGFP and pCMV-LMP1/EGFP vectors contained the EGFP gene from pEGFP-N1 (BD Biosciences Clontech) inserted into the pcDNA3.1-Mat and pcDNA3.1-LMP1 vectors.

Western blot analysis of EGFP, GST, HBsAg, p53, MMP-7, EBV-LMP1, and AKT1. At 48 h after transfection, cells were directly lysed on 6-well culture plates in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 2 mM EDTA, and 1% NP-40) containing protease inhibitors (Roche, Mannheim, Germany). Total protein extracts were separated on a 12% SDS–polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore, Billerica, MA, USA), and incubated with anti-GFP (B-2), anti-GST (B-14), anti-c-Myc (9E10) or anti-p53 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), sheep polyclonal anti-HBsAg antibodies (Serotec, Oxford, UK), anti-EBV-LMP1 CS 1-4 monoclonal antibody (DakoCytomation, Inc., Carpinteria, CA, USA) or rabbit polyclonal anti-AKT1 antibodies (Cell Signaling Technology Inc., Beverly, MA, USA) followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), anti-sheep IgG secondary antibody (abcam, Cambridgeshire, UK) or anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). The bands were detected by using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Results

Strategy and experimental design for screening and identifying effective RNAi probes

To screen and identify the effective RNAi probes more robustly and cost-effectively, we have developed a reliable and quantitative reporter-based siRNA validation system required only a short synthetic DNA fragment. This system is composed of a short synthetic DNA fragment and two expression vectors for targeting reporter and triggering siRNA expression (Fig. 1). The short synthetic DNA fragment is formed by annealing two complementary sense- and antisense-oligonucleotides, which contain a unique RNAi targeting sequence with 19-nt in length flanked by five consecutive adenosine and thymidine residues (As and Ts) at the 5′ and 3′ ends as an effective termination signal for transcription of the antisense- and sense-RNA, respectively (Fig. 1B). The unique RNAi targeting sequence is selected and designed from protein coding region of the target gene according to the sequence-specific characteristics of the effective siRNAs as described in Introduction. To construct the targeting reporter vector, a short synthetic DNA fragment containing a unique RNAi targeting sequence of interest is fused with a reporter gene at the 5′-, 3′-UTR or is inserted within the reporter gene without interfering in its activity (Fig. 1A). To make the greatest value and utility of this short synthetic DNA fragment, simultaneously, it is also cloned into a specific triggering siRNA expression vector that contains two functional convergent RNA Pol III promoters (Fig. 1C). Efficacy of the siRNAs is measured by their abilities to inhibit

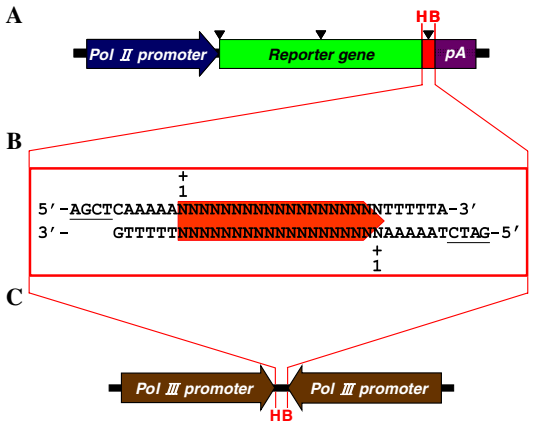


Fig. 1. Strategy and experimental design for screening effective RNAi probes using reporter-based siRNA validation system. This reporter-based siRNA validation system is composed of a short synthetic DNA fragment (B) and two expression vectors for targeting reporter (A) and triggering siRNA (C) expression. The short synthetic DNA fragment contains a unique RNAi targeting sequence with 19-nt in length and two specific restriction enzyme *Hin*dIII and *Bgl*II compatible ends for inserting into the *Hin*dIII/*Bgl*II-digested targeting reporter and triggering siRNA expression vectors simultaneously. The restriction enzyme compatible ends of *Hin*dIII (5′-AGCT) and *Bgl*II (5′-GATC) are underlined. The targeting reporter vector not only contains the reporter gene expression cassette driven by RNA Pol II promoter but also includes two unique restriction enzyme sites, *Hin*dIII (H) and *Bgl*II (B), for construction of the RNAi targeting sequence at the 5′- or 3′-UTR or insertion within the reporter gene without interfering its activity. The positions for inserting the RNAi targeting sequence are marked with black triangles. The triggering siRNA expression vector contains two convergent RNA Pol III promoters to drive expression of both the sense and antisense strands of the siRNA, respectively. The short synthetic DNA fragment is also cloned into the *Hin*dIII (H) and *Bgl*II (B) sites in triggering siRNA expression vector.

expression of the targeting reporter gene, which contains the corresponding short synthetic DNA fragment, with easily quantified readouts including EGFP or firefly luciferase. In addition, to make this system complete, it is convenient to include an excellent *in vitro* cell model, which not only provides an easy cell culture system but also has high transfection efficiency. Because this system is used for screening the highly effective RNAi probes directly against genes with wide range of biological functions, it is better to perform the screening in a non-human and non-mouse cell line. To fit these criteria, the baby hamster kidney fibroblast BHK is chosen as an *in vitro* experimental model. Moreover, to standardize the experimental procedures, including cell culture conditions and transfection protocols, have been optimized.

Construction of the reporter-based siRNA validation system including targeting reporter and triggering siRNA expression vectors

This reporter-based siRNA validation system includes two expression vectors for targeting reporter and triggering siRNA expression (Figs. 1A and C). To simply normalize the transfection variation and accurately evaluate the efficacy of the RNAi probes, the targeting reporter vectors all contain two independent expression cassettes for transcription of the targeting reporter (EGFP or firefly luciferase) and reference protein (glutathione *S*-transferase, GST or *Renilla* luciferase) genes (Fig. 2A). The simple and sensitive EGFP-fluorescence detection or luciferase assay combined with well-documented and easily analyzed reference protein, GST or *Renilla* luciferase, provides an easy and reliable readout for the system. In addition, to test the silencing effects of an RNAi targeting sequence located at different positions on the targeting reporter gene, we have constructed three distinct targeting reporter vectors that contain two unique restriction enzyme *Hin*dIII and *Bgl*II sites at either the 5′-UTR or 3′-UTR of the reporter gene or within a chimeric fusion reporter gene for constructing the RNAi targeting sequence (Fig. 2A).

To fully utilize the short synthetic DNA fragment for producing the triggering siRNA, we have constructed a particular siRNA expression vector, pDual (Fig. 2B), which contains two convergent RNA Pol III promoters, mouse U6 and human H1, to drive expression of both the sense and antisense strands of siRNA by using the short synthetic DNA fragment as template, respectively. In addition, to simply and efficiently clone the short synthetic DNA fragment containing the RNAi targeting sequence of interest into this vector, the pDual vector also contains the same *Hin*dIII and *Bgl*II restriction enzyme sites located between mouse U6 and human H1 promoters in which the sense and antisense strands of siRNA are transcribed by U6 and H1 promoters, respectively. Previous studies have shown that the shRNA exhibits slightly better effect on the inhibition of gene expression as compared with that of the siRNA [17,38]. To make this study complete, we have also constructed a highly effective shRNA expression vector, pHsH1 (Fig. 2B), which contains only the human H1 promoter to drive transcription of the consecutive sequence of the sense, a loop, and the antisense regions.

In addition, to objectively and accurately determine the efficacy of selected RNAi targeting sequence mediated inhibition of the targeting reporter gene expression in both the EGFP- and firefly luciferase-based siRNA validation systems, it is essential to have highly effective RNAi probes directly against EGFP and firefly luciferase expression. For this purpose, we have particularly constructed four effective shRNA and siRNA expression vectors for inhibiting EGFP (pHsH1-shEGFP and pDual-siEGFP) and firefly luciferase (pHsH1-shLuc and pDual-siLuc) expression that could serve as references for positive controls. These effective RNAi probes target on the coding regions of EGFP and firefly luciferase mRNA transcripts, and exhibit high inhibition effects with a silencing efficiency of more than 90%.

Evaluation of the reporter-based siRNA validation system with well-analyzed siRNAs

To evaluate the performance and accuracy of this system, we have first examined this system with two

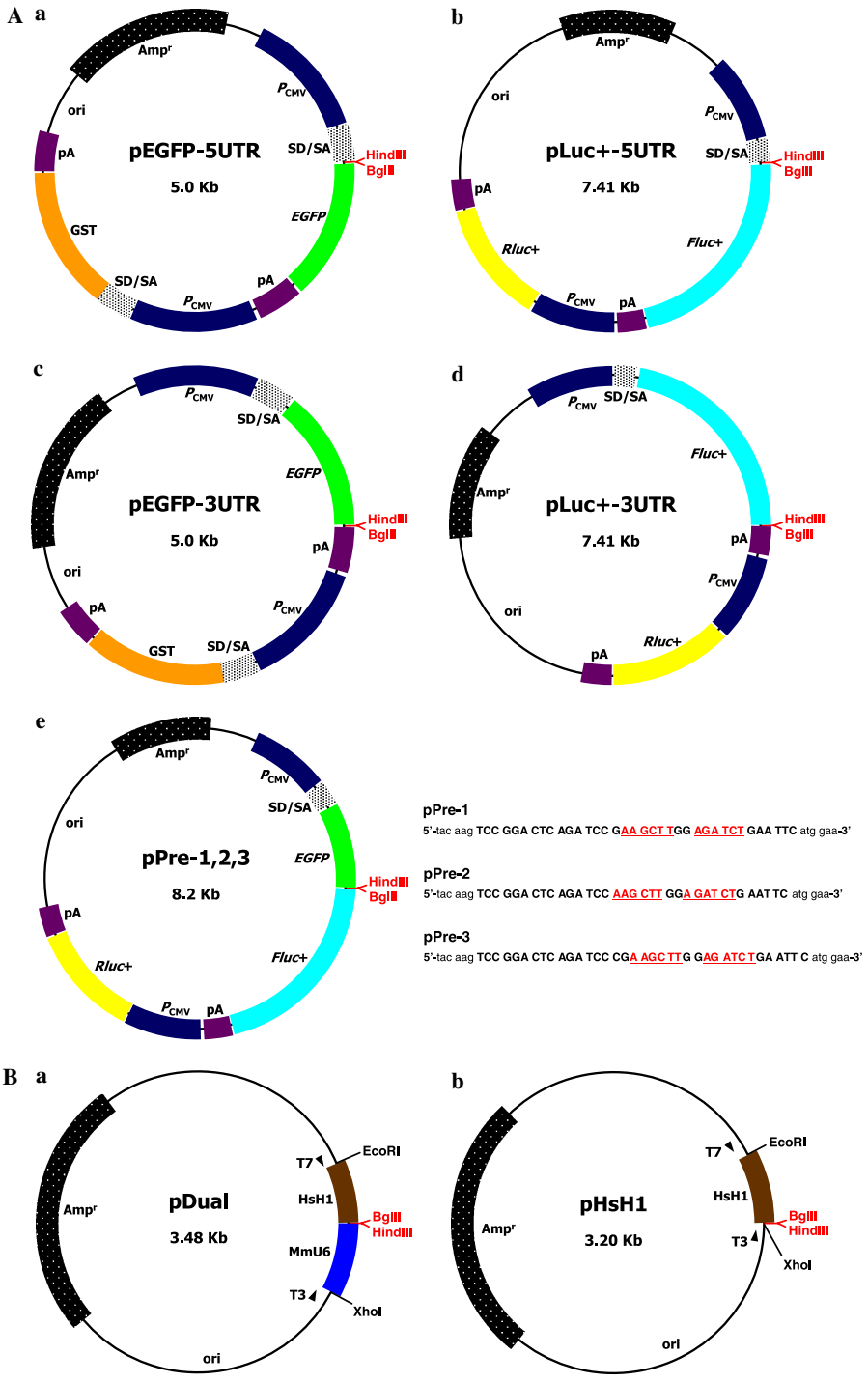


Fig. 2. Targeting reporter and triggering siRNA expression vectors. (A) Constructs of targeting reporter expression vectors. The vectors contain two independent expression cassettes for expression of the targeting reporter and reference protein as well as two unique restriction enzyme sites, *Hin*dIII and *Bgl*II, located either at the 5′-UTR (a and b) or 3′-UTR (c and d) of the reporter gene or within a chimeric fusion reporter gene (e) for constructing the RNAi targeting sequence. The unique restriction enzyme sites, *Hin*dIII and *Bgl*II, for constructing the RNAi targeting sequence are in red. pEGFP-5UTR (a) and pEGFP-3UTR (c) contain the *EGFP* as targeting reporter and *GST* as reference protein. pLuc+-5UTR (b) and pLuc+-3UTR (d) contain the firefly luciferase (*Fluc*+) as targeting reporter and *Renilla* luciferase (*Rluc*+) as reference protein. pPre-1, 2, and 3 (e) three vectors contain the chimeric EGFP-firefly luciferase fusion protein (*EGFP-Fluc*+) as targeting reporter and *Renilla* luciferase as reference protein, as well as they possess three different reading frame sequences between *EGFP* and *Fluc*+ fused sites for inserting RNAi targeting sequences with different reading frames. (B) Constructs of triggering siRNA expression vectors. The siRNA expression plasmid pDual (a) contains two convergent RNA Pol III promoters, mouse U6 (*MmU6*) and human H1 (*HsH1*), to drive expression of both the sense and antisense strands of the siRNA, respectively. The shRNA expression plasmid pHsH1 (b) contains only the human H1 (*HsH1*) promoter to drive transcription of the consecutive sequence of the sense, a loop, and the antisense regions.

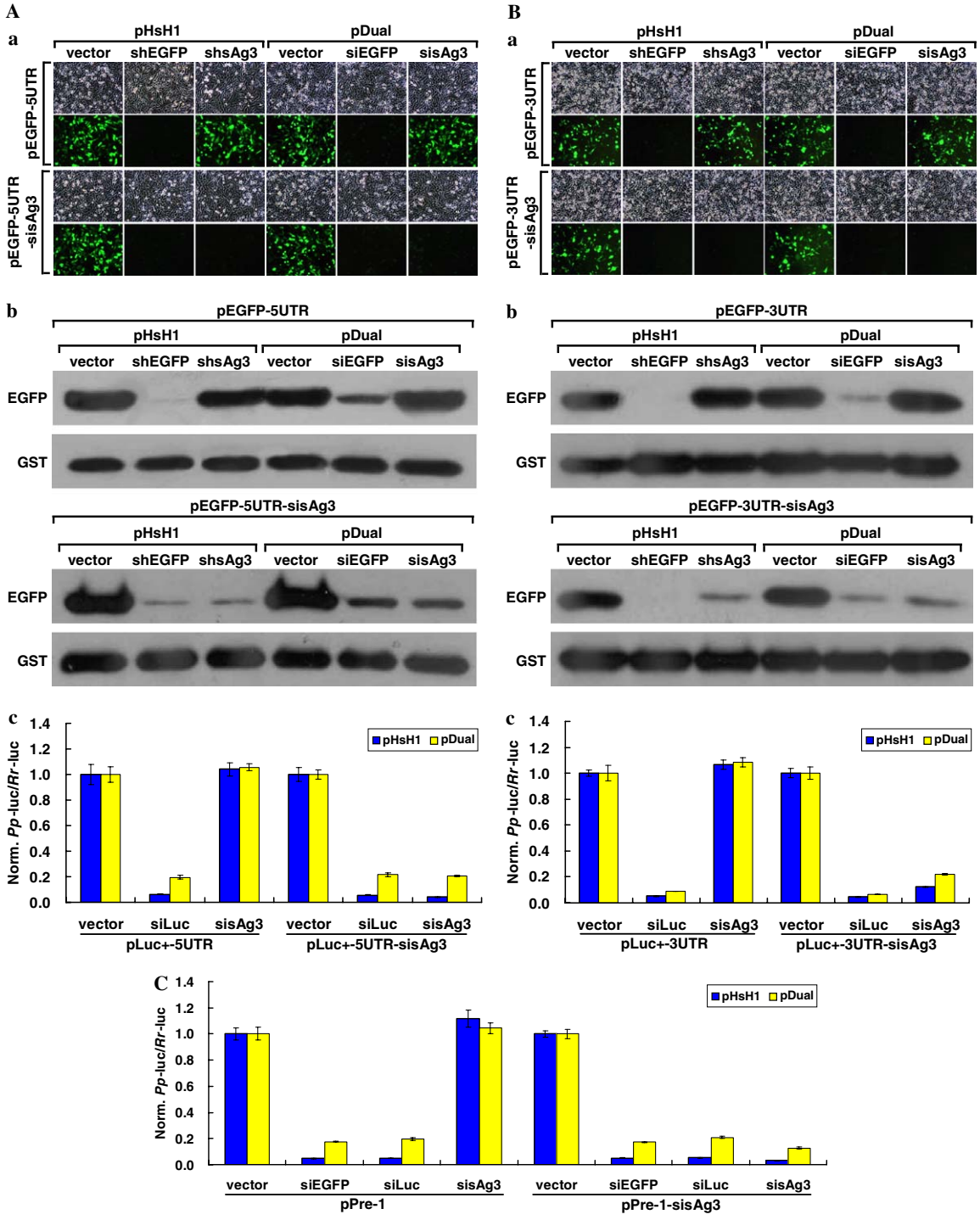


Fig. 3. Effects of active RNAi targeting sequence sisAg3 mediated inhibition of the targeting reporter expression. Inhibition effects of (A) 5′-UTR-sisAg3- or (B) 3′-UTR-sisAg3-mediated targeting reporter gene expression in BHK cells. The cells were cotransfected with 0.5 μg of targeting reporter expression vector (pEGFP-5UTR-sisAg3 or 3UTR-sisAg3; pLuc+-5UTR-sisAg3 or 3UTR-sisAg3) and 1.5 μg of triggering siRNA expression vector (pHsH1-shsAg3, shEGFP or shLuc; pDual-sisAg3, siEGFP or siLuc) as indicated by Lipofectamine 2000. At 48 h posttransfection, the cells expressed EGFP were examined by inverted fluorescent microscopy (a); the expression levels of EGFP and GST in the total protein extracts were further determined by Western blot analysis (b); and the expression levels of firefly and *Renilla* luciferases in the total protein extracts were measured by Dual-luciferase Reporter Assay System (c). (C) Inhibition effects of sisAg3-mediated targeting chimeric fusion reporter gene expression in BHK cells. The cells were cotransfected with 0.5 μg of targeting reporter expression vector pPre-1-sisAg3 and 1.5 μg of triggering siRNA expression vector (pHsH1-shsAg3, shEGFP or shLuc; pDual-sisAg3, siEGFP or siLuc) as indicated by Lipofectamine 2000. At 48 h posttransfection, the expression levels of chimeric EGFP-firefly luciferase fusion protein (EGFP-Fluc+) and *Renilla* luciferase in the total protein extracts were measured by Dual-luciferase Reporter Assay System. The firefly luciferase/*Renilla* luciferase (*Pp-luc*/*Rr-luc*) ratio was normalized and calculated against the control vector (pHsH1 or pDual). The plotted data were averaged from three independent experiments and the bars indicate standard deviation. The levels of GST serve as reference protein for loading control.

well-analyzed RNAi targeting sequences, sisAg1 and sisAg3, on HBV-HBsAg gene. Our previous studies have shown that the sisAg3 exhibits an extremely potent silencing effect on HBsAg expression, while the sisAg1 displays totally no inhibition effect [37]. To determine the inhibition effects of pHsH1-expressed shsAg1 (or shsAg3) and pDual-expressed sisAg1 (or sisAg3) on HBsAg expression, human hepatoma cell line Huh7 cells were cotransfected with HBsAg expression vector p(3A)SAg-luc+ or p(3A)SAg-EGFP and shsAg expression vector pHsH1-shsAg1 (or shsAg3) or sisAg expression vector pDual-sisAg1 (or sisAg3). The expression levels of HBsAg, firefly luciferase, and EGFP in total protein extracts were determined at 48 h posttransfection. Consistent with the results obtained from the previous studies, not only the pHsH1-expressed shsAg3 but also the pDual-expressed sisAg3 induced an extremely high inhibition effect on p(3A)SAg-luc+- and p(3A)SAg-EGFP-induced HBsAg expression; in contrast, both the shsAg1 and sisAg1 dsRNAs had no inhibition effect on HBsAg expression as compared to the vector only (Fig. S1A). These results clearly indicated that both the shRNA and siRNA expressed from pHsH1 and pDual vectors, respectively, could effectively induce gene silencing with similar efficacy.

To simply test the efficiency of this system in reporting the efficacy of an inactive sisAg1- and active sisAg3-mediated inhibition of targeting reporter expression, we have fused the sisAg1 or sisAg3 RNAi targeting sequence with EGFP or firefly luciferase reporter gene at the 5′-, 3′-UTR or inserted between EGFP-Fluc+ fusion gene. The *in vitro* cell model BHK cells were cotransfected with targeting reporter expression vector (pEGFP-5UTR-sisAg3 or 3UTR-sisAg3; pLuc+-5UTR-sisAg3 or 3UTR-sisAg3; pPre-1-sisAg3) and triggering siRNA expression vector (pHsH1-shsAg3 or pDual-sisAg3). At 48 h posttransfection, the expression levels of EGFP, GST, firefly, and *Renilla* luciferases in total protein extracts were analyzed. When the RNAi targeting sequence sisAg3 was inserted at the 5′- or 3′-UTR of EGFP gene, both the pHsH1-expressed shsAg3 and pDual-expressed sisAg3 induced strong inhibition of EGFP production (Figs. 3A and B). In addition, when the reporter gene EGFP was replaced by firefly luciferase, both the shsAg3 and sisAg3 exhibited similar inhibition effects on the 5′- or 3′-UTR sisAg3-mediated targeting reporter firefly luciferase expression (Figs. 3A and B). Further, we have repeated the experiments with the RNAi targeting sequence sisAg3 inserted within the chimeric EGFP-Fluc+ fusion gene. As the results shown in Fig. 3C, both the shsAg3 and sisAg3 induced strong inhibition on EGFP-Fluc+ fusion gene expression according to the suppression of firefly luciferase activity. These results indicated that the active RNAi targeting sequence sisAg3 could strongly mediate inhibition of targeting reporter EGFP, firefly luciferase or EGFP-Fluc+ chimeric protein production.

To check whether this system could really reflect the efficacy of selected RNAi targeting sequences, we have tested this system with the inactive RNAi targeting sequence sisAg1. BHK cells were cotransfected with targeting reporter expression vector (pEGFP-5UTR-sisAg1 or 3UTR-sisAg1; pLuc+-5UTR-sisAg1 or 3UTR-sisAg1; pPre-1-sisAg1) and triggering siRNA expression vector (pHsH1-shsAg1 or pDual-sisAg1). The expression levels of EGFP, GST, firefly, and *Renilla* luciferases in total protein extracts were determined at 48 h posttransfection. As compared with the results obtained from using the active RNAi targeting sequence sisAg3, the inactive sisAg1 totally had no inhibition effects on targeting reporter gene expression in all tested systems including sisAg1 fused at the 5′- or 3′-UTR of EGFP or firefly luciferase or inserted within EGFP-Fluc+ fusion gene, as well as pHsH1-expressed shsAg1 and pDual-expressed sisAg1 (Fig. S2).

To further confirm the efficiency of this system in reporting the efficacy of selected RNAi targeting sequences, we have repeatedly examined this system with another important and well-documented RNAi targeting sequence sip53 on tumor suppressor protein p53 gene. Previous studies have shown that this sip53 could efficiently induce inhibition of p53 expression in either the transient, stable or inducible gene silencing experiment [18,38,39]. To determine the inhibition effect of sip53 expressed from pDual vector, BHK cells were cotransfected with p53 expression vector pCMV-p53/EGFP and shp53 expression vector pHsH1-shp53 or sip53 expression vector pDual-sip53. At 48 h posttransfection, the expression levels of p53 and EGFP in total protein extracts were analyzed. As expected, the pDual-expressed sip53 also displayed strong inhibition effect on pCMV-p53/EGFP-induced p53 expression as compared to that of pHsH1-expressed shp53 (Fig. S1B).

To further examine the efficiency of this system in reporting the efficacy of active sip53 targeting sequence, BHK cells were cotransfected with targeting reporter expression vector (pEGFP-5UTR-sip53 or 3UTR-sip53; pLuc+-5UTR-sip53 or 3UTR-sip53; pPre-1-sip53) and triggering siRNA expression vector (pHsH1-shp53 or pDual-sip53). At 48 h posttransfection, the expression levels of EGFP, GST, firefly, and *Renilla* luciferases were determined. As the results shown in Fig. S3, the active sip53 almost copied the inhibition effects of sisAg3-mediated targeting reporter gene expression in all experimental conditions. Taken together, these results clearly demonstrated that this reporter-based siRNA validation system could effectively and faithfully report the efficacy of selected RNAi targeting sequences, as well as that all three distinct targeting reporter constructs exhibited similar efficiency in reporting the efficacy of selected RNAi probes.

Application of this reporter-based siRNA validation system to identify effective siRNAs

To simply and efficiently perform this reporter-based siRNA validation system, we have only focused on the targeting reporter expression vectors, pEGFP-3UTR and pLuc+-3UTR, and the triggering siRNA expression

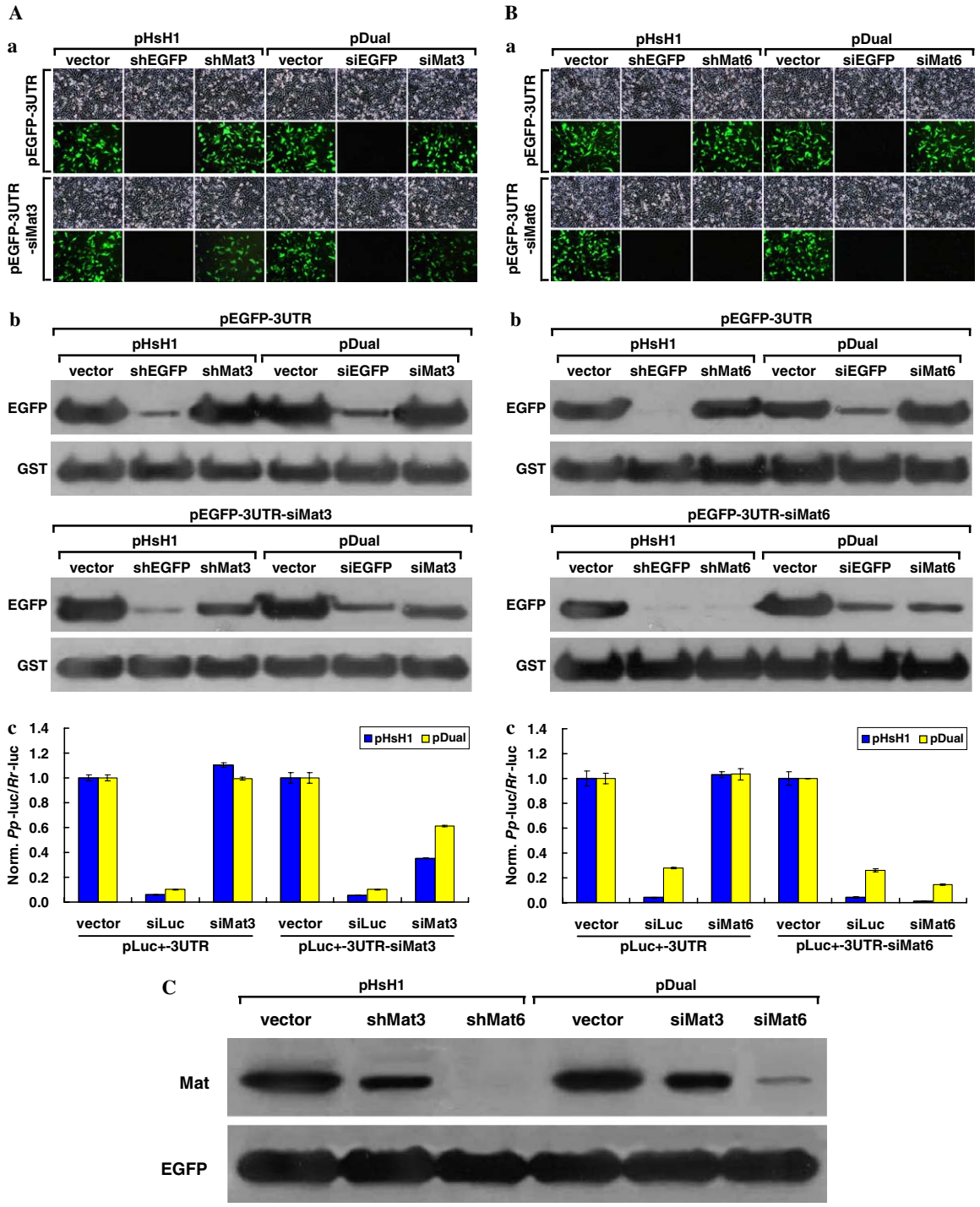


Fig. 4. Effects of selected RNAi targeting sequences of mouse MMP-7, siMat3, and siMat6 mediated inhibition of the targeting reporter expression. Inhibition effects of (A) 3′-UTR-siMat3- and (B) 3′-UTR-siMat6-mediated targeting reporter gene expression in BHK cells. The cells were cotransfected with 0.5 μg of targeting reporter expression vector (pEGFP-3UTR-siMat3 or siMat6; pLuc+-3UTR-siMat3 or siMat6) and 1.5 μg of triggering siRNA expression vector (pHsH1-shMat3, shMat6, shEGFP or shLuc; pDual-siMat3, siMat6, siEGFP or siLuc) as indicated by Lipofectamine 2000. At 48 h posttransfection, the cells expressed EGFP were examined by inverted fluorescent microscopy (a); the expression levels of EGFP and GST in the total protein extracts were further determined by Western blot analysis (b); and the expression levels of firefly and *Renilla* luciferases in the total protein extracts were measured by Dual-luciferase Reporter Assay System (c). The firefly luciferase/*Renilla* luciferase (*Pp-luc*/*Rr-luc*) ratio was normalized and calculated against the control vector (pHsH1 or pDual). The plotted data were averaged from three independent experiments and the bars indicate standard deviation. The levels of GST serve as reference protein for loading control. (C) Inhibition effects of siMat3 and siMat6 on MMP-7 expression in BHK cells. The cells were cotransfected with 0.5 μg pCMV-Mat/EGFP and 1.5 μg of triggering siRNA expression vector (pHsH1-shMat3 or shMat6; pDual-siMat3 or siMat6) as indicated by Lipofectamine 2000. At 48 h posttransfection, the expression levels of MMP-7 and EGFP in the total protein extracts were determined by Western blot analysis. The levels of EGFP serve as reference protein for transfection efficiency.

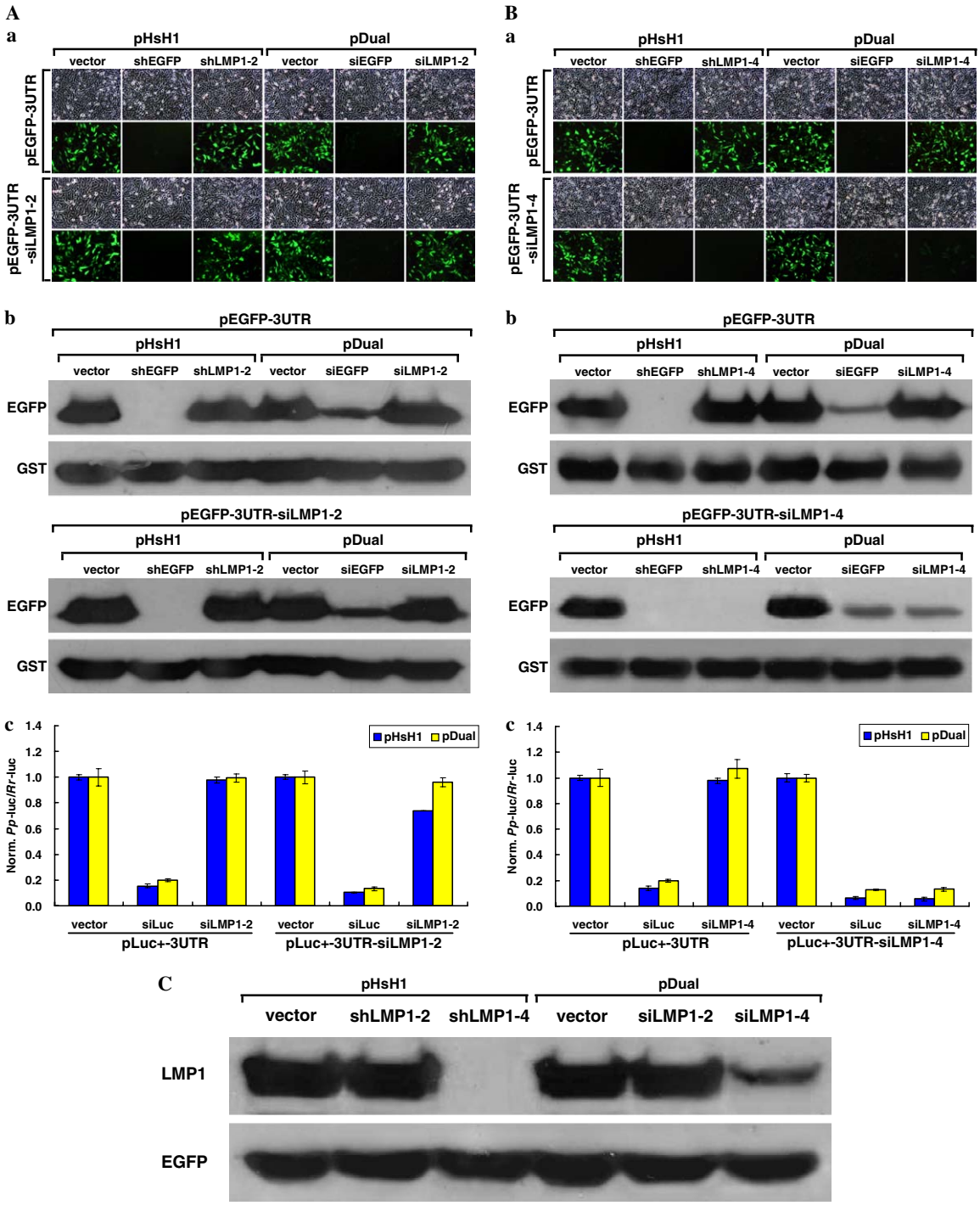


Fig. 5. Effects of selected RNAi targeting sequences of EBV-LMP1, siLMP1-2, and siLMP1-4 mediated inhibition of the targeting reporter expression. Inhibition effects of (A) 3′-UTR-siLMP1-2- and (B) siLMP1-4-mediated targeting reporter gene expression in BHK cells. The cells were cotransfected with 0.5 μg of targeting reporter expression vector (pEGFP-3UTR-siLMP1-2 or siLMP1-4; pLuc+-3UTR-siLMP1-2 or siLMP1-4) and 1.5 μg of triggering siRNA expression vector (pHsH1-shLMP1-2, shLMP1-4, shEGFP or shLuc; pDual-siLMP1-2, siLMP1-4, siEGFP or siLuc) as indicated by Lipofectamine 2000. At 48 h posttransfection, the cells expressed EGFP were examined by inverted fluorescent microscopy (a); the expression levels of EGFP and GST in the total protein extracts were further determined by Western blot analysis (b); and the expression levels of firefly and *Renilla* luciferases in the total protein extracts were measured by Dual-luciferase Reporter Assay System (c). The firefly luciferase/*Renilla* luciferase (*Pp-luc*/*Rr-luc*) ratio was normalized and calculated against the control vector (pHsH1 or pDual). The plotted data were averaged from three independent experiments and the bars indicate standard deviation. The levels of GST serve as reference protein for loading control. (C) Inhibition effects of siLMP1-2 and siLMP1-4 on EBV-LMP1 expression in BHK cells. The cells were cotransfected with 0.5 μg pCMV-LMP1/EGFP and 1.5 μg of triggering siRNA expression vector (pHsH1-shLMP1-2 or shLMP1-4; pDual-siLMP1-2 or siLMP1-4) as indicated by Lipofectamine 2000. At 48 h posttransfection, the expression levels of LMP1 and EGFP in the total protein extracts were determined by Western blot analysis. The levels of EGFP serve as reference protein for transfection efficiency.

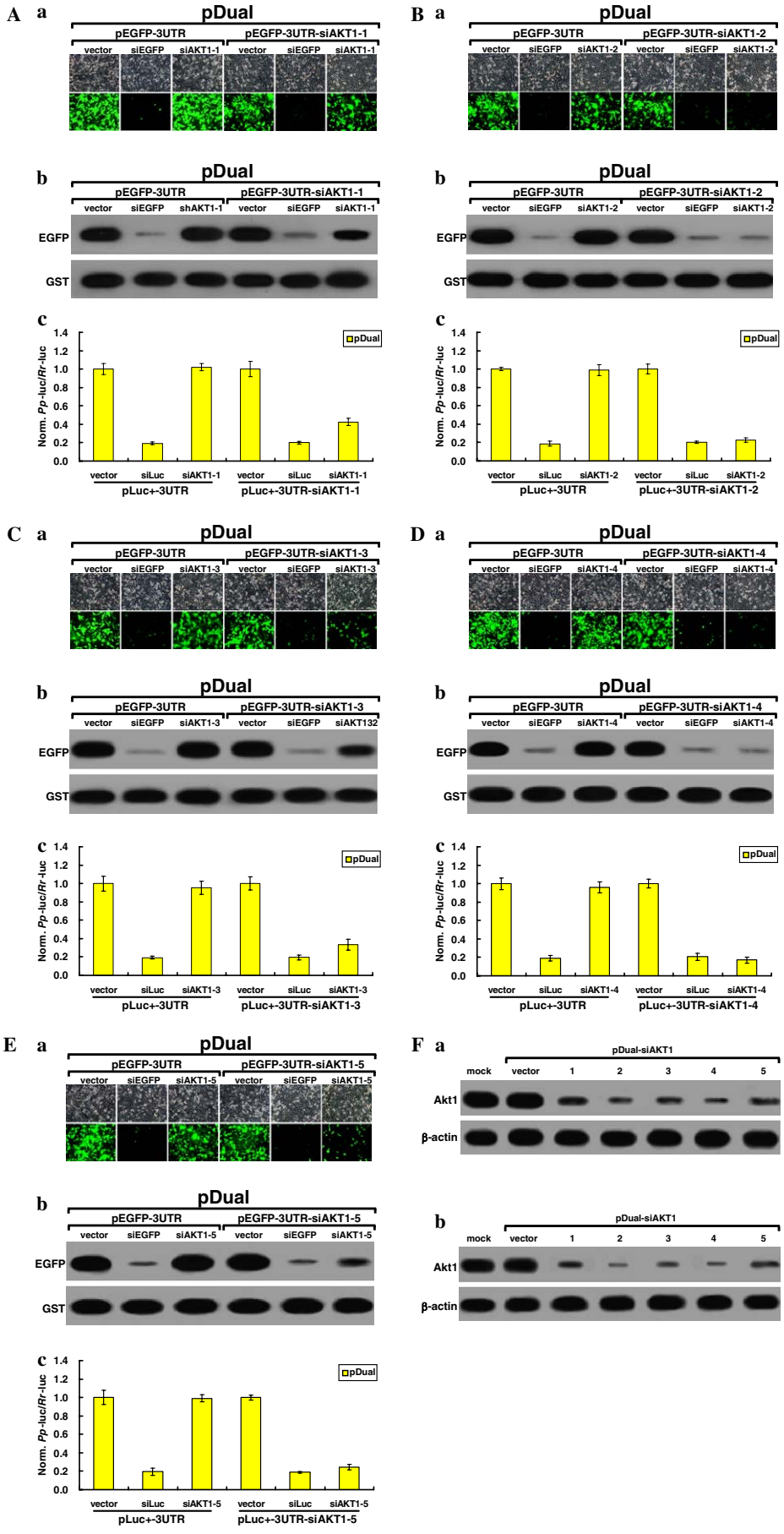
vector, pDual. To actually test this system for selecting effective RNAi targeting sequences of interest, we have employed this system to identify highly effective RNAi probes directly against mouse MMP-7, EBV-LMP1, and human AKT1. MMP-7, one of the smallest members of the matrix metalloproteinase (MMP) family, not only associates with tumor progression and metastasis but also plays an essential role in innate immunity in particular inflammation disorders [40]. It has been shown that MMP-7 might contribute to tumor resistance to cytotoxic agents and suggested that inactivation of MMP-7 might enhance the efficacy of conventional cancer chemotherapy [40].

To directly apply this reporter-based siRNA validation system for identifying effective siRNAs, we have designed and screened two putative RNAi probes targeting the coding sequence of mouse MMP-7. BHK cells were cotransfected with targeting reporter expression vector (pEGFP-3UTR-siMat3 or siMat6; pLuc+-3UTR-siMat3 or siMat6) and triggering siRNA expression vector (pHsH1-shMat3 or shMat6; pDual-siMat3 or siMat6). The expression levels of EGFP, GST, firefly, and *Renilla* luciferases were determined at 48 h posttransfection. As the results shown in Fig. 4A, the siMat3 only induced partial inhibition effects on expression of the targeting reporter EGFP or firefly luciferase expressed from pEGFP-3UTR-siMat3 or pLuc+-3UTR-siMat3, respectively. In contrast with the siMat3, the siMat6 mediated strong inhibition effects as compared with two standard positive controls, siEGFP and siLuc, expressed from either pHsH1 or pDual (Fig. 4B). According to the results indicated, the RNAi targeting sequence siMat6 exhibited inhibition effect with efficacy more than 90%. To further confirm these results, BHK cells were cotransfected again with MMP-7 expression vector pCMV-Mat/EGFP and triggering siRNA expression vector (pHsH1-shMat3 or shMat6; pDual-siMat3 or siMat6). Consistent with the results obtained from the reporter-based siRNA validation system, the selected active shMat6 and siMat6 expressed from pHsH1 and pDual vectors, respectively, indeed exhibited high inhibition effects on pCMV-Mat/EGFP-induced MMP-7 expression, in contrast, the partially active shMat3 and siMat3 had inhibition effects with efficacy about 40–50% (Fig. 4C).

EBV, a human γ-herpesvirus, is not only able to establish life-long and asymptomatic infection in the vast majority of individuals worldwide but also highly associated with an increasing number of tumors and lymphoid diseases [41]. The EBV-induced pathogenesis is strongly associated with viral proteins that contribute to cell immortalization and immune evasion. Previous studies have shown that disruptions of these viral proteins resulted in inhibition of either viral replication or transforming abilities, suggesting that these proteins are potential candidates as therapeutic targets [42,43]. The LMP1 functions as a constitutively activated member of the tumor necrosis factor receptor (TNFR) superfamily and activates several signaling pathways in a ligand-independent manner, which induces several of genes that encode anti-apoptotic proteins and cytokines. LMP1 not only involves in type II and type III EBV latency but also regards as an oncoprotein in rodent fibroblast transformation and EBV-induced B cell immortalization.

To explore further the potential of this method, we have additionally designed and screened two siRNAs targeting the coding sequence of EBV-LMP1. BHK cells were cotransfected with targeting reporter expression vector (pEGFP-3UTR-siLMP1-2 or siLMP1-4; pLuc+-3UTR-siLMP1-2 or siLMP1-4) and triggering siRNA expression vector (pHsH1-shLMP1-2 or shLMP1-4; pDual-siLMP1-2 or siLMP1-4). At 48 h posttransfection, the expression levels of EGFP, GST, firefly, and *Renilla* luciferases were determined. As the results shown in Fig. 5A, the siLMP1-2 exhibited no inhibition effects on pEGFP-3UTR-siLMP1-2-induced EGFP and pLuc+-3UTR-siLMP1-2-induced firefly luciferase expression as compared with the two standard positive controls, siEGFP and siLuc, indicating that the selected RNAi targeting sequence siLMP1-2 was inactive and non-functional. In contrast with inactive siLMP1-2, the siLMP1-4 exhibited strong inhibition effects on expression of the EGFP and firefly luciferase expressed from pEGFP-3UTR-siLMP1-4 and pLuc+-3UTR-siLMP1-4 targeting reporter expression vectors, respectively (Fig. 5B). As compared with the efficacy of two standard positive controls, siEGFP and siLuc, the evaluated efficacy of siLMP1-4 was more than 90%. To directly assess the efficacy of siLMP1-2 and siLMP1-4 in

Fig. 6. Effects of selected RNAi targeting sequences of AKT1, including siAKT1-1, siAKT1-2, siAKT1-3, siAKT1-4, and siAKT1-5, mediated inhibition of the targeting reporter and AKT1 expression. Inhibition effects of (A) 3′-UTR-siAKT1-1-, (B) siAKT1-2-, (C) siAKT1-3-, (D) siAKT1-4-, and (E) siAKT1-5-mediated targeting reporter gene expression in BHK cells. The cells were cotransfected with 0.5 μg of targeting reporter expression vector (pEGFP-3UTR-siAKT1-1, siAKT1-2, siAKT1-3, siAKT1-4 or siAKT1-5; pLuc+-3UTR-siAKT1-1, siAKT1-2, siAKT1-3, siAKT1-4 or siAKT1-5) and 1.5 μg of triggering siRNA expression vector (pDual-siAKT1-1, siAKT1-2, siAKT1-3, siAKT1-4 or siAKT1-5, siEGFP or siLuc) as indicated by Lipofectamine 2000. At 48 h posttransfection, the cells expressed EGFP were examined by inverted fluorescent microscopy (a); the expression levels of EGFP and GST in the total protein extracts were further determined by Western blot analysis (b); and the expression levels of firefly and *Renilla* luciferases in the total protein extracts were measured by Dual-luciferase Reporter Assay System (c). The firefly luciferase/*Renilla* luciferase (*Pp-luc*/*Rr-luc*) ratio was normalized and calculated against the control vector (pDual). The plotted data were averaged from three independent experiments and the bars indicate standard deviation. The levels of GST serve as reference protein for loading control. (F) Inhibition effects of siAKT1-1, siAKT1-2, siAKT1-3, siAKT1-4 and siAKT1-5 on endogenous AKT expression in HeLa (a) and HEK293 (b) cells. Both the cells were transfected with 2 μg of triggering siRNA expression vector (pDual-siAKT1-1, siAKT1-2, siAKT1-3, siAKT1-4 or siAKT1-5) as indicated by Lipofectamine 2000. At 48 h posttransfection, the expression levels of AKT1 and β-actin in the total protein extracts were determined by Western blot analysis. The levels of β-actin serve as reference protein for loading control.


A
a
pDual
pEGFP-3UTR
pEGFP-3UTR-siAKT1-1
vector
siEGFP
siAKT1-1
b
shAKT1-1
EGFP
GST
c
pDual
Norm. Pp-luc/Rr-luc
siLuc
pLuc+-3UTR
pLuc+-3UTR-siAKT1-1
B
pEGFP-3UTR-siAKT1-2
siAKT1-2
pLuc+-3UTR-siAKT1-2
C
pEGFP-3UTR-siAKT1-3
siAKT1-3
siAKT132
pLuc+-3UTR-siAKT1-3
D
pEGFP-3UTR-siAKT1-4
siAKT1-4
pLuc+-3UTR-siAKT1-4
E
pEGFP-3UTR-siAKT1-5
siAKT1-5
pLuc+-3UTR-siAKT1-5
F
pDual-siAKT1
mock
1
2
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Akt1
β-actin

inhibition of EBV-LMP1 expression, BHK cells were repeatedly cotransfected with LMP1 expression vector pCMV-LMP1/EGFP and triggering siRNA expression vector (pHsH1-shLMP1-2 or shLMP1-4; pDual-siLMP1-2 or siLMP1-4). As the predicted, the shLMP1-4 and siLMP1-4 induced strong inhibition effects on pCMV-LMP1/EGFP-induced LMP1 production, while both the shLMP1-2 and siLMP1-2 were totally ineffective (Fig. 5C).

The phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway plays a critical role in cell proliferation and survival. Perturbations of this signaling pathway have been found in a variety of human cancer cells. Previous studies have shown that constitutively activated PI3K/AKT signaling pathway is sufficient to induce oncogenic transformation of cells and tumor formation in transgenic mice [44]. In addition, inhibition of this signaling pathway results in growth inhibition and apoptosis of cancer cells with in particular elevated this signaling activity. Thus, blocking the hyperactive PI3K/AKT signaling pathway provides a new strategy for targeted cancer therapy, especially for cancer cells whose survival and growth are dominated by constitutively active PI3K/AKT signaling [45].

To additionally evaluate the authenticity of this reporter-based siRNA validation system, we have particularly designed and screened five RNAi targeting sequences on the coding region of human AKT1. BHK cells were cotransfected with targeting reporter expression vector (pEGFP-3UTR-siAKT1-1, siAKT1-2, siAKT1-3, siAKT1-4 or siAKT1-5; pLuc+-3UTR-siAKT1-1, siAKT1-2, siAKT1-3, siAKT1-4 or siAKT1-5) and triggering siRNA expression vector (pDual-siAKT1-1, siAKT1-2, siAKT1-3, siAKT1-4 or siAKT1-5). The expression levels of EGFP, GST, firefly, and *Renilla* luciferases were determined at 48 h posttransfection. As the results shown in Figs. 6A, C, and E, the siAKT1-1, siAKT1-3, and siAKT1-5 only induced low to medium levels of inhibition on expression of the targeting reporter EGFP or firefly luciferase expressed from pEGFP-3UTR-siAKT1-1, siAKT1-3 or siAKT1-5; pLuc+-3UTR-siAKT1-1, siAKT1-3 or siAKT1-5. In contrast with the siAKT1-1, siAKT1-3, and siAKT1-5, the siAKT1-2 and siAKT1-4 triggered strong inhibition effects as compared with two standard positive controls, siEGFP and siLuc, expressed from pDual (Figs. 6B and D). According to the results indicated, the RNAi targeting sequences siAKT1-2 and siAKT1-4 exhibited inhibition effect with efficacy more than 90%. To further confirm these results, HeLa and HEK293 cells were transfected with triggering siRNA expression vector, pDual-siAKT1-1, siAKT1-2, siAKT1-3, siAKT1-4 or siAKT1-5. Consistent with the results obtained from the reporter-based siRNA validation system, the selectively active siAKT1-2 and siAKT1-4 expressed from pDual indeed exhibited high inhibition effects on endogenous AKT1 expression; in contrast, the partially active siAKT1-1, siAKT1-3, and siAKT1-5 had inhibition effects with efficacy of about 60–70% (Fig. 6F). These results clearly indicated that this reporter-based siRNA validation system could robustly and efficiently screen highly effective RNAi probes for either reverse functional genomics or gene silencing-based therapeutics.

Discussion

In this study, we established a reliable and quantitative reporter-based siRNA validation system, and also demonstrated that this system could effectively and faithfully report the efficacy of the corresponding siRNA in a sequence-specific manner. This system is composed of two expression vectors for targeting reporter and triggering siRNA expression, as well as two highly effective siRNAs, siEGFP [46] and siLuc [38], which serve as references for positive controls. In addition, to make this system more comparative, either the sisAg3 [37] or sip53 [18] could also be included as a positive reference that could effectively and specifically mediate inhibition of the targeting reporter EGFP or firefly luciferase expression via the corresponding RNAi targeting sequence. As compared with the inhibition levels of these positive references, one could easily evaluate the efficacy of designed RNAi probes of interest.

In comparison with the previous methods [34–36], the only requirement for this system is a short synthetic DNA fragment that contains a unique RNAi targeting sequence with 19-nt in length. This short synthetic DNA fragment can be used for constructing both the targeting reporter and triggering siRNA expression vectors simultaneously. It has been shown that fusion of the targeting reporter gene with the different length of RNAi targeting sequence either 19- or 38-nt exhibits no significant difference on inhibition effects induced by the corresponding siRNAs [35]. These results clearly indicate that the 19-nt sequence in an active siRNA is enough to serve as a functional RNAi targeting sequence for achieving the reporter-based siRNA validation assay. As an ideal and workable system, the RNAi targeting sequence is conveniently and efficiently fused at the 3′-UTR of the targeting reporter gene with no regard to its open reading frame and no effect on its translation. On the basis of this principle, the targeting reporter expression vectors, pEGFP-3UTR and pLuc+-3UTR, provide a complete system for constructing any RNAi targeting sequences of interest.

There are many approaches to build the profile of a particular gene and its function. One of the best and simple ways to investigate gene function is to disrupt or knock down the gene and determine the phenotype of the resulting mutant. To efficiently apply RNAi technology for reverse functional genomics [8–11], in particular the novel or putative genes with only available nucleotide sequences in databases but without cDNA clones in hand, one could simply identify the effective RNAi probes directly against the novel or putative genes by using this system. Once the highly effective RNAi probes are identified, one could easily establish the specific gene knockdown in the *in vitro* cell lines or *in vivo* animal models that provide a loss-of-function mutation in the novel or putative genes.

Subsequently, many different molecular, cellular, biochemical, and other analyses could be performed to examine the inhibition effects on the *in vitro* or *in vivo* models.

siRNAs could be used clinically to inhibit gene expression as a therapeutic agent in many diseases characterized by elevated gene function. Inhibition of virus-specific genes by siRNAs has proven to be a potential therapeutic strategy against virus induced diseases. For instance, inhibition of virus replication and gene expression by directly introducing siRNAs into the cells has been reported for numerous viruses [47,48], including several important human pathogens such as poliovirus [49], HIV-1 [22,50], hepatitis C virus [51], influenza virus [52], dengue virus [53], and hepatitis B virus [37,54,55]. A number of extremely virulent viruses including Ebola, Lassa, severe acute respiratory syndrome (SARS), avian influenza (H5N1), West Nile, and smallpox viruses are highly infectious and cause extraordinarily deadly diseases [56,57]. Furthermore, there are currently no vaccines or effective therapies available, and in particular these viruses require special containment for safe research. To develop an extremely potent RNAi-based therapeutics for these virulent viruses with safety, this reporter-based siRNA validation system could provide a simple and powerful approach for screening and identification of highly effective siRNAs directly against viral gene expression without the need of direct virus culture. Currently, we have successfully screened and identified several highly effective RNAi probes potentially against these virulent viruses by simply using the rational design strategy and this reporter-based siRNA validation system (C.-F. Teng, unpublished data).

In summary, our results clearly demonstrate that this reporter-based siRNA validation system not only could effectively and faithfully report the efficacy of the corresponding siRNAs in a sequence-specific manner but also provided a simple and cost-effective system for simultaneously constructing both the targeting reporter and triggering siRNA expression vectors with only requirement of a short synthetic DNA fragment as compared with the previous approaches [35,36]. Although previous studies have described the usefulness of the reporter-based siRNA validation method in screening effective siRNAs, our studies advanced its capacities not only for using in high-throughput application but also for reverse genetic studies of novel gene function. Because of its simplicity and effectiveness, this approach is useful for large-scale analysis of mammalian gene function.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.05.164.

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