BMB
reports

Multiple shRNA expressing vector enhances efficiency of gene silencing

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RNA interference (RNAi) is the process of sequence-specific gene silencing. However, RNAi efficiency still needs to be improved for effective inhibition of target genes. We have developed an effective strategy to express multiple shRNAs (small hairpin RNA) simultaneously using multiple RNA Polymerase III (Pol III) promoters in a single vector. Our data demonstrate that multiple shRNAs expressed from Pol III promoters have a synergistic effect in repressing the target gene. Silencing of endogenous cyclophilin A (CypA) or key HIV viral genes by multiple shRNAs results in significant inhibition of the target gene. [BMB reports 2008; 41(5): 358-362]

INTRODUCTION

RNAi technology has several limitations, in spite of being a powerful tool for gene silencing. Unlike gene knockout that results in 100% inhibition of the target gene, RNAi efficiency can, at best, reach 80-90% inhibition of the target gene (1, 2). Leakage in RNAi-mediated gene silencing may significantly decrease the effect of targeting; the transcripts that escape from RNAi can partially recover its normal function. Pol III promoter-based multiple shRNAs have been used to enhance the efficiency of RNAi (3). However, effective vector construction techniques are still not available since cloning of multiple transcription units into one vector is a challenge. We have overcome this technical hurdle and developed an effective strategy for constructing an RNAi vector that silences multiple sites simultaneously. Our data demonstrate that multiple targeting RNAi has a synergistic effect on the repression of a target, and can produce a near-knockout effect.

RESULTS AND DISCUSSION

A simple strategy for construction of a multiple-site targeting RNAi vector

We have developed an effective approach to constructing a multiple-site targeting RNAi vector, pMultiRNAi (Fig. 1). We generated a plasmid, p2RNAi-donor, as template DNA for PCR. p2RNAi-donor contains two RNA polymerase III promoters (H1 and U6) in opposing orientations (Fig. 1A). We used two different promoters in the p2RNAi-donor plasmid to prevent formation of a secondary structure of DNA. The secondary structure of DNA reduces the efficiency of PCR, as well as subsequent cloning.

We have designed PCR primers that contain the following elements, in order: a promoter sequence, a target sequence, a loop sequence, a complementary sequence, a termination signal, and a cloning site (Fig. 1B). Vector construction strategy consists of two straightforward steps (Fig. 1C). In the first step, we used PCR to obtain the DNA fragments that contain multiple promoters and multiple target sites. In the second step, we ligated the PCR products to a cloning vector to generate pMultiRNAi. Using this strategy, it is possible to target two, four, or even six different target sites at once.

We tested our construction strategy using randomly selected target sites: A, B, C, and D of a murine gene for cyclophilin A (CypA). pMultiRNAi-CypA-AB and pMultiRNAi-CypA-CD were constructed with each plasmid containing two target sites. PCR was performed to obtain the promoters and targeting (Fig. 1D). The PCR fragments were digested with *Hind*III and *Eco*RI (pMultiRNAi-CypA-AB), *Eco*RI and *Xho*I (pMultiRNAi-CypA-CD), respectively, then were ligated into the cloning sites (*Hind*III and *Xho*I) of the cloning vector to form pMultiRNAi-CypA-AB and pMultiRNAi-CypA-CD. All six colonies screened contained the inserted fragment, indicating a high subcloning efficiency (Fig. 1E). The presence of the insert fragments was further confirmed by sequencing (data not shown).

Our strategy also provides flexibility for construction. First, other restriction sites can be used as cloning sites to substitute the above sites to fit the need of the experiments. Second, because the promoter and the RNAi elements were located in the PCR fragments, any plasmid containing two appropriate cloning sites can be used as the backbone vector.

Synergistic effect of RNAi with two target sites

We first examined the RNAi efficiency of a single target site versus that of two target sites. RNAi vectors that target CypA at a single or two sites (A, B, C, D; AB and CD) were transfected

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Received 20 November 2007, Accepted 20 December 2007

Keywords: Gene silencing, RNA interference, RNAi, shRNA, Vector

into p19 cells. An RNAi vector targeting a non-specific sequence (pRNAi-NS) was used as a control. Western blotting analysis was performed to detect the expression of CypA (Fig. 2A). The single-target sites (A, B, C, and D) showed inhibitory effects at various levels, while the combination of two target sites (AB and CD) had significantly stronger inhibitory effects. We further quantified the protein levels of the target gene to confirm the synergistic effect of multiple target sites. The relative protein levels of CypA for target sites A, B, and the combination of the two sites (AB) were 27%, 38%, and 13%, respectively. Similarly, the relative protein levels of CypA for target sites C, D, and the combination of the two sites (CD) were 38%, 62%, and 25%, respectively (Fig. 2B). These experiments demonstrated that RNAi had a synergistic effect when two sites were targeted.

Gene silencing by multiple RNAi targeting

We extended our construction strategy to include four target sites. Four primers were used in two PCR sets (Fig. 3A). The two PCR fragments were digested with *Hind*III and *Eco*RI, *Eco*RI and *Xho*I, then were ligated into cloning sites (*Hind*III and *Xho*I) of the vector (Fig. 3B). We constructed pMultiRNAi-CypA to target four sites (A, B, C, and D) of the murine CypA gene, and examined the RNAi effect. The multiple-site targeting (ABCD) had a much stronger RNAi effect (Fig. 3C). We further quantified the protein levels of the target gene to confirm the synergistic effect of multiple target sites. The relative protein levels of CypA for the combination of the four sites was 2% (Fig. 3D). These experiments therefore demonstrated that RNAi had a synergistic effect when multiple sites were targeted.

We then examined the biological effects of the knockdown of CypA. We previously found that CypA is required for retinoic acid-induced neuronal differentiation of p19 cells (4). We examined the expression β-tubulin III, a marker for neuronal differentiation in different knockdown cells with or without retinoic acid treatment. The expression of β-tubulin III was decreased in cells transfected with a single-targeting vector (site B), and was not detectable in the cells transfected with the multiple-targeting vector (four sites) (Fig. 3E). This indicates that retinoic acid-induced differentiation of p19 cells correlated with the expression levels of CypA.

RNAi is often used in targeting viruses (5, 6). We then examined the effect of multiple RNAi in the repression of viral replication. HeLa/CD4+ cells were co-transfected using the HIV-$1_{NL4.3}$ expression plasmid, pNL4.3, and one of the following RNAi vectors as indicated in Fig. 3F. Real-time PCR was used to detect the mRNA levels of rev gene. Our data demonstrated a synergistic RNAi effect when multiple sites were targeted (Fig. 3F). We also examined whether multiple shRNAs cause cell toxicity. Because cell toxicity usually affects cell proliferation, we monitored the proliferation of the above cells 48 hr post-transfection (Fig. 3G). Our data showed that cell

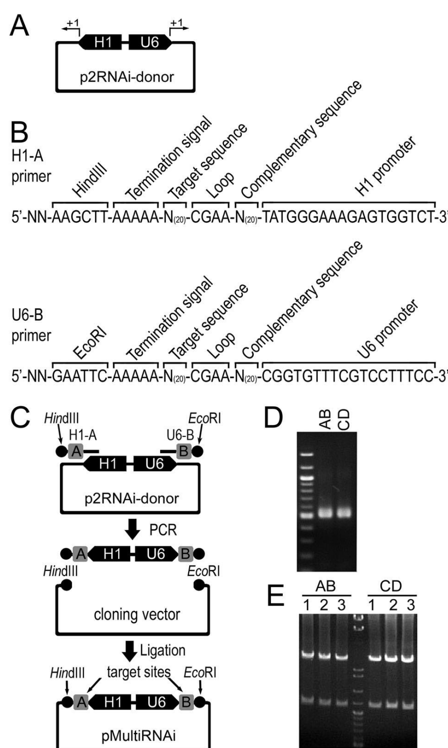

Fig. 1. Construction strategy of the multiple-targeting RNAi vector, pMultiRNAi. (A) Map of the p2RNAi-donor plasmid. The transcription initiation sites for H1 and U6 promoters are indicated as +1. (B) Primer design for PCR. (C) Construction of vector pMultiRNAi. H1 and U6, promoters; A and B, target sites; H1-A and U6-B, PCR primers; *Hind*III and *Eco*RI, cloning sites. (D) PCR using two long primers. AB and CD, RNAi target sites. (E) Screening of the positive colonies. AB and CD, RNAi target sites.

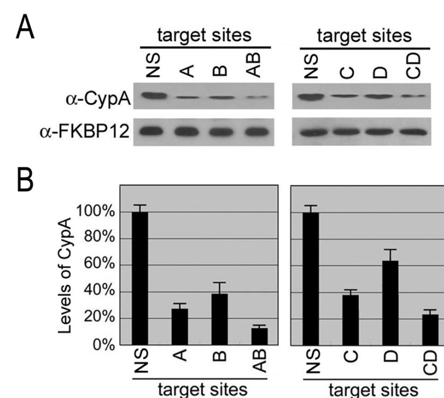

Fig. 2. Gene silencing by two-site targeting. (A) Gene silencing of CypA by RNAi vectors that target a single site or a combination of two sites. Protein level of FKBP12 was used as a control. NS, non-specific target site. (B) The synergistic effect of RNAi targeting two sites. Protein level of CypA in control cells (NS) was taken as 100%. The results are from three identical experiments.

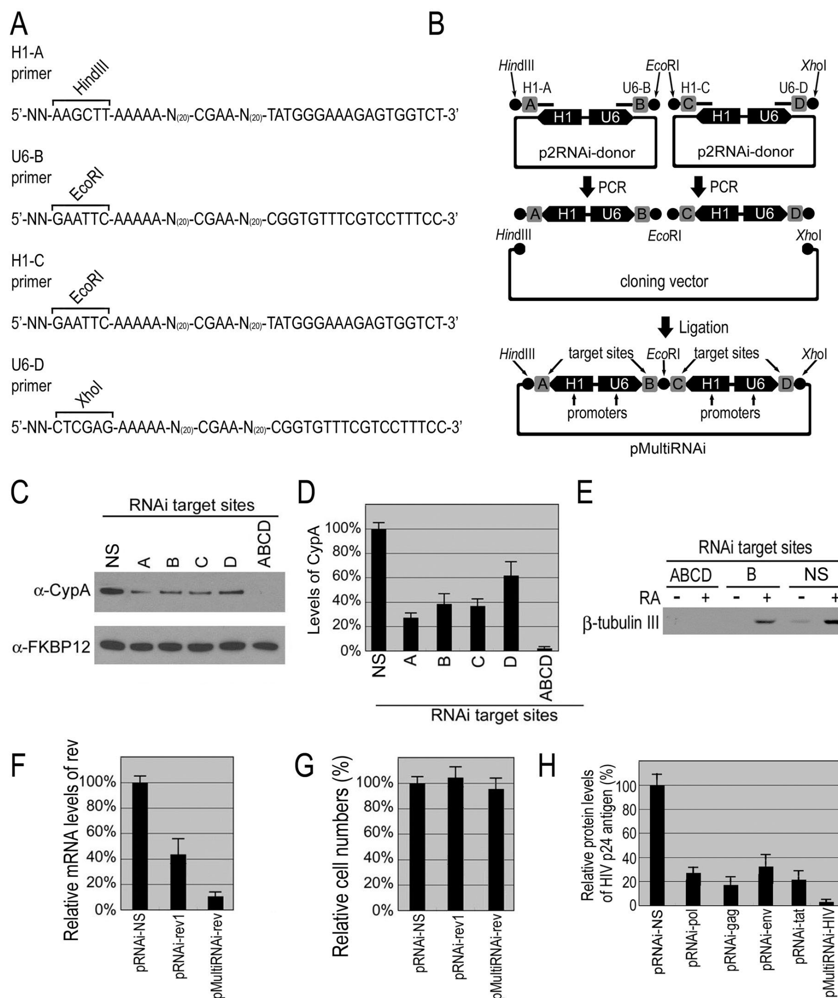

Fig. 3. Gene silencing by four-site targeting. (A) Primer design for PCR. (B) Construction of vector. H1 and U6, promoters; A, B, C, and D, target sites; H1-A, U6-B, H1-C, and U6-D, PCR primers; *Hind*III, *Eco*RI, and *Xho*I, cloning sites. (C) Gene silencing of CypA by RNAi vectors that target a single site or the combination of four sites. Protein level of FKBP12 was used as a control. NS, non-specific target site. (D) The synergistic effect of RNAi targeting multiple sites. Protein level of CypA in control cells (NS) was taken as 100%. The results are from three identical experiments. (E) Retinoic acid-induced differentiation of p19 cells with different protein levels of CypA. (F) Multiple silencing of HIV rev gene. Relative mRNA levels were indicated. (G) Proliferation of cells with RNAi vector expressing single or multiple shRNAs. (H) Inhibition of HIV-1 replication by single or multiple gene silencing. The results are from three identical experiments.

proliferation was not affected by multiple shRNAs compared to that of single shRNAs. The result indicates that multiple shRNAs can inhibit target gene without additional cell toxicity.

We also tested the effect of silencing multiple HIV viral genes. HeLa/CD4+ cells were co-transfected using the HIV-$1_{NL4.3}$ expression plasmid, pNL4.3, and one of the following RNAi vectors as indicated in Fig. 3H. The viral p24 antigen levels were quantified by ELISA. The levels of p24 were reduced to ~15-30% in samples with single targeting, but were dramatically reduced to 4% in the samples with multiple targeting (Fig. 3H), suggesting that multiple gene silencing can more effectively inhibit replication of HIV-1 than single gene silencing.

Several companies such as Invitrogen and Ambion have produced a combination of synthetic multiple siRNAs that target distinct regions of the same gene to facilitate effective degradation of the target. The strategy, termed RNAi pooling, increases the chances of reducing target gene expression by the random-designed siRNAs in the short term. Recent report has demonstrated that vector-based multiple shRNAs have great potential for inhibiting their target genes (3). Vector-based multiple RNAi had greater advantage in gene silencing because of its long term effect. Our approach solved the problem of the

construction of the multiple RNAi vector, thus made it possible to express multiple shRNAs from multiple promoters in an efficient manner. On the other hand, miRNA-based approaches with targeting of multiple sites were reported (7, 8). It will be of interest to compare the efficiencies and specificities of multiple-site targeting between shRNA and miRNA.

The multiple targeting RNAi vector can not only be used to enhance the efficiency of RNAi, but can also target multiple genes. Most biological processes, such as tumorigenesis, signal transduction, and differentiation involve multiple genes. Thus, multiple targeting RNAi vectors would significantly improve the control of these processes.

MATERIALS AND METHODS

Plasmids

To construct p2RNAi-donor, the H1 and U6 promoters were ligated into pBluescript II KS+ (Stratagene, CA, USA). The U6 promoter was obtained from pNeoRNAi (4). The H1 promoter was obtained by PCR using the following primers: H1F (5′-TATAAGCTTGGATCCCCAAGGAATCGCGGGCCCAG-3′) and H1R 5-TATAGTCGACTATTCCATGGGAAAGAGTGGTCT CATACAGAA-3′.

To generate pMultiRNAi-CypA, p2RNAi-donor plasmid was used as template DNA using the following two sets of primers: H1-A (5′-ATAAGCTTAAAAAGACTTTAC-ACGCCATAATGGC GAACCATTATGGCGTGTAAAGTCTATGGGAAAGAGTGGT CT-3′) and U6-B (5′-ATGAATTCAAAAAGACCAAACACAAAC GGTTCCCGAA-GGAACCGTTTGTGTTTGGTCCGGTGTTTCG TCCTTTCC-3′); H1-C (5′-ATGAA-TTCAAAAAGACAAAGTTCC AAAGACAGCCGAAGCTGTCTTTGGAACTTTGTCTATGGGA AAGAGTGGTCT-3′) and U6-D (5′-ATCTCGAGAAAAAGACT GAA-TGGCTGGATGGCACGAATGCCATCCAGCCATTCAGT CCGGTGTTTCGTCCTTTCC-3′). PCR products were double-digested with either *Hind*III and *Eco*RI or *Eco*RI and *Xho*I, depending on the cloning sites of the PCR products. The purified fragments were cloned into pBluescript II KS+ to generate pMultiRNAi-CypA. Similarly, pMultiRNAi-HIV was generated using the following two sets of primers: H1-pol (5′-ATAAGCTT AAAAATGCTCCTGTATCTAATAGAGCCGAAGCTCTATTAGA TACAGGAGCATATGGGAAAGAGTGGTCT-3′) and U6-gag (5′-ATGAATTCAAAAA-GTTCTAGCTCCCTGCTTGCCCCGAAGG GCAAGCAGGGAGCTAGAACCGGTGTTTCGTCCTTTCC-3′); U6-env (5′-ATCTCGAGAAAAAGATGTGGCAGGAAGTA-GG ACCGAAGTCCTACTTCCTGCCACATCCGGTGTTTCGTCCTT TCC-3′) and H1-tat (5′-ATGAATTCAAAAACTGCTTGTACCAA TTGCTATTCGAAAATAGCAATT-GGTACAAGCAGTATGGGA AAGAGTGGTCT-3′). Single-target vectors pRNAi-CypA-A, pRNAi-CypA-B, pRNAi-CypA-C, pRNAi-CypA-D, pRNAi-HIV-pol, pRNAi-HIV-gag, pRNAi-HIV-tat, and pRNAi-HIV-env were also constructed as follows: The RNAi elements (H1 or U6 promoter and one target site) were obtained using PCR. p2RNAi-donor plasmid was used as template DNA using H1 or U6 forward primer and reverse primers that containing one target site. The PCR fragments were then cloned into pBluescript II KS+ to form the single targeting vectors. Similarly, pMultiRNAi-rev (four sites) and pRNAi-HIV-rev1 were also constructed. Target sites for rev gene are 5′-GGCACTTATCTGGGACGAT-3′ (rev1), 5′-GGTAG CTGAAGAGGCACAGGC-3′ (rev2), 5′-GAGGGGACCAGGGA GAGCAC-3′ (rev3), and 5′-ACTTACTCTTGATT-GTAAC-3′ (rev4). An RNAi vector targeting a non-specific sequence (pRNAi-NS) was generated as a control. The randomly generated scramble sequence contained in pRNAi-NS does not target human or mouse genes.

Cell culture and transfection

Plasmids were transfected into p19 cells and HeLa/CD4+ cells. p19 cells were grown in Minimum Essential Medium Alpha Medium (MEMα) supplemented with 7.5% bovine calf serum and 2.5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). HeLa/CD4+ cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS. Cells were maintained on 12-well plates and transfected using Lipofectamine 2000 (Invitrogen) according to the protocol provided by the manufacturer.

Western blotting analysis

Western blotting analysis was used to detect RNAi-mediated inhibition of target genes. Whole-cell lysates were electrophoresed and immunoblotted according to the protocol provided by Santa Cruz Biotechnology, Inc. Antibodies used were rabbit anti-CypA (BIOMOL Res Lab Inc., PA, USA) and rabbit anti-FKBP12 (Affinity Bioreagents, Inc., CO, USA). Anti-CypA was used to detect the target. Anti-FKBP12 was used as an internal control. FKBP12 is a ubiquitously expressed protein belonging to the immunophilin family in mammalian cells (9). A secondary antibody mouse anti-rabbit-IgG-horseradish peroxidase (HRP) was used to detect the primary rabbit antibodies. The signals were detected using ECL reagents (Pierce, Rockford, IL, USA). The data was quantitated by densitometric measurements of the signals on the x-ray film.

p24 enzyme-linked immuno-sorbent assay (ELISA)

The culture supernatants were collected post-transfection and analyzed by ELISA. HIV-1 p24 ELISA Kit (Cat. # NEK050B001KT, PerkinElmer, Shelton, CT, USA) was used as instructed in the protocol provided by the manufacturer.

Real-time PCR

Real-time PCR was performed using ABI Prism 7700 Sequence Detection System (Perkin Elmer). Primers for rev gene are 5′-CAGCGACGAAGAGCTCATCA-3′ and 5′-TCACTAATCGAAT GGATCTG-3′. The PCR cycle conditions were 95°C for 4 min followed by 45 cycles of 95°C for 15 sec and 60°C for 30 sec. All samples were run in triplicates in the same culture plate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as a normalization control.

Acknowledgements
The authors are grateful to Kristen Lum for critical review of the manuscript.

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