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RESEARCH ARTICLE

A simple and visualized method to screen for effective siRNAs by using green fluorescence protein (GFP) as a reporter

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Abstract To screen for effective small interference RNA (siRNA), a simple and visualized method was developed using the green fluorescence protein (GFP) as a reporter. Candidate siRNAs targeting macrophage migration inhibition factor genes (MIF) were identified. By using the p*EGFP-N*3 vector, the *MIF-GFP* expression plasmid, p*EGFP-MIF*, was constructed with the same Kozak consensus translation initiation site and start code ATG for the MIF-EGFP coding sequence. Based on the siRNA expression vector pSilencer-4.1, 3 candidate MIF siRNA expression plasmids were constructed and co-transfected with the p*EGFP-MIF* into the HEK293 cells, respectively. The GFP expression in HEK293 cells could be viewed by fluorescence microscopy and the *MIF* mRNA expressions were determined by real-time quantitative PCR. The 3 candidate *MIF* siRNA expression plasmids were also co-transfected with the *MIF* expression plasmid into the HEK293 cells, respectively, and the *MIF* mRNA expressions were determined by real-time quantitative PCR. The results show that the down-regulated expression of the *MIF* mRNA was consistent with the GFP expression and the same effective *MIF* siRNAs were screened by using the p*EGFP-MIF* or *MIF* expression plasmid with the candidate *MIF* siRNAs expression plasmids. Therefore, by using the GFP as a reporter, a useful method was provided to screen for effective siRNAs targeting specific genes co-expressed with the *GFP*. This may be a good strategy for screening for effective siRNAs targeting different genes.

Keywords RNA interference, real-time quantitative PCR, green fluorescence protein, vector

1 Introduction

RNA interference (RNAi) is a process of sequence specific gene silencing post transcription mediated by double-strand RNA. The exogenous double-strand RNA was recognized by the endonuclease Dicer and dissected into 21 to 25 nt small double-strand RNA (siRNA). The siRNA can incorporate into RNA-induced silencing complexes (RISC), and then the target mRNA could be cut at the complementary region of the anti-sense strand of siRNA mediated by the RISC. Thus, the expression of the target gene could be specifically suppressed (Matzke et al., 2001).

Presently, the RNAi technique has been widely used in the study of gene function, cancer and gene therapy. Since one target gene can be silenced by several siRNAs to different extents, it is important to screen for and identify the effective siRNAs for gene function and related studies. In the current study, by using the macrophage migration inhibition factor gene (*MIF*) as a target gene, a simple and visualized method to screen for effective siRNAs by using green fluorescence protein (GFP) as a reporter was introduced.

2 Materials and methods

2.1 Plasmid and cell line

The linearized small hairpin RNA (shRNA) expression vector pSilencer-4.1-neo, which was digested by the *Bam*H I/*Hin*d III and pSilencer-Negative control vector were purchased from the Ambion Company (USA). The green fluorescence protein (GFP) expression plasmid was purchased from the Clontech Company (USA). The plasmid T-GAPDH and MIF expression plasmid pSec-tag-MIF were constructed by colleagues in our laboratory. The *E. coli* DH5α and HEK293 cell line were stored in our laboratory.

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2.2 Construction of MIF-GFP fusion expression plasmid

The PCR primers were designed and synthesized according to the sequences of multi-cloning sites in the p*EGFP*-N3 vector (GenBank Accession number: U57609), the coding sequence of the enhanced GFP (EGFP) and MIF gene (GenBank Accession number: NM_002415). The sequence scheme of the MIF-GFP expression plasmid to be constructed is shown in Figure 1. The forward primer for *MIF* gene was 5′- GCCAAGCTTCGCCACCATGGTGATGCCGATGTTCATCGTAAA-3′, including the *Hin*d III recognition sequence and the Kozak consensus translation initiation site CGCCACCATGG. The reverse primer for the *MIF* gene was 5′- CCAGGATCCGGCGAAGGTGGAGTTGTT-3′, including the *Bam*H I recognition sequence. Based on the DNA template of the pSec-tag-*MIF*, the *MIF* gene was amplified. The PCR was performed for 2 min at 94°C followed by 31 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 40 s, with an auto-extension for 5 min at 72°C after the completion of the last cycle. The forward primer for the *EGFP* was 5′-GAAGGATCCGTGAGCAAGGGCGAGGAG-3′, including the *Bam*H I recognition sequence. The reverse primer was 5′-AGTCGCGGCCGCTTACTTGTACAGCTCGTCCA-3′, including the *Not* I recognition sequence. Based on the DNA template of p *EGFP*-N3, the *EGFP* gene was amplified with the same PCR condition as above. Then, the *MIF* gene fragment was digested by the *Hin*d III/*Bam*HI, the *EGFP* gene fragment was digested by the *Bam*H I/*Not* I, and the p *EGFP*-N3 vector was digested by the *Hin*d III/*Not* I, respectively. The three digested products were purified and used for ligation. The ligation product was transfected into the chemically competent *E. coli*. DH5α. The transfected cells were spread on the LB solid medium plates containing 50 μg/mL kanamycin. The plates were incubated at 37°C overnight. The positive clones were selected and cultured in the LB liquid medium containing 50 μg/mL kanamycin. The plasmid DNAs of the positive clones were extracted and identified by restriction enzymes and DNA sequencing analysis.

2.3 Construction of the MIF siRNAs expression plasmids

Three candidate siRNAs targeting MIF gene were selected by using the siRNA designing programme of the Ambion Company (USA). The DNA templates for the MIF siRNAs and EGFP siRNA were annealed by two complimentary oligonucleotide strands. The loop sequence of the two DNA templates was TTCAAGAGA, with the complimentary sequences at the bilateralis and two sticky end sequences corresponding to the terminations of the linearized pSilencer-4.1-*neo*. According to the targeting sequence of the MIF siRNA1, CAGGGTCTACATCAACTATTA, two single oligonucleotide strands were designed and synthesized. The sequence of the sense strand was 5′-GATCCGGGTCTACATCAACTATTATTCAAGAGATAATAGTTGATGTAGACCCTGA-3′, and the anti-sense strand was 5′-AGCTTCAGGGTCTACATCAACTATTATCTCTTGAATAATAGTTGATGTAGACCCG-3′. Another two single oligonucleotide strands were designed and synthesized according to the targeting sequence of the MIF siRNA2, CAACTATTACGACATGAACGC. The sequence of the sense strand was 5′-GATCCACTATTACGACATGAACGCTTCAAGAGAGCGTTCATGTCGTAATAGTTGA-3′, and the anti-sense strand was 5′-AGCTTCAACTATTACGACATGAACGCTCTCTTGAAGCGTTCATGTCGTAATAGTG-3′. The corresponding two single oligonucleotide strands were designed and synthesized according to the targeting sequence of the MIF siRNA3, CAACTCCACCTTCGCCTAAGA. The sequence of the sense strand was 5′-GATCCACTCCACCTTCGCCTAAGATTCAAGAGATCTTAGGCGAAGGTGGAGTTGA-3′, and the anti-sense strand was 5′-AGCTTCAACTCCACCTTCGCCTAAGATCTCTTGAATCTTAGGCGAAGGTGGAGTG-3′.

Two single oligonucleotide strands were designed and synthesized according to the targeting sequence of the *GFP* siRNA, CGGCAAGCTGACCCTGAAGTTCAT[3]. The sequence of the sense strand was 5′-GATCCGCAAGCTGACCCTGAAGTTCATTTCAAGAGAATGAACTTCAGGGTCAGCTTGCCGA-3′, and the anti-sense strand was 5′-AGCTTCGGCAAGCTGACCCTGAAGTTCATTCTCTTGAAATGAACTTCAGGGTCAGCTTGCG-3′. The corresponding two single oligonucleotide strands were denaturated at 94°C for 30 min and annealed at 37°C for 1 h to obtain a double stranded DNA fragment. The annealed DNA fragment was introduced into the linearized plasmid pSilencer-4.1-*neo* using a high efficiency ligation reagent. The ligation product was transfected into the chemically competent *E. coli*. DH5α, and then the transfected cells were spread on the LB solid

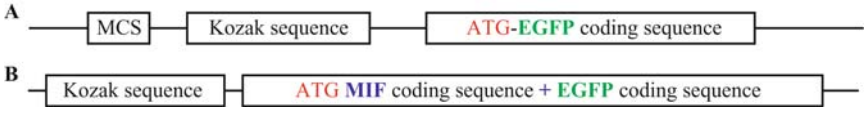

Fig. 1 Structures of the partial pEGFP-N_3 and MIF-GFP expression vector. A: the structure of partial pEGFP-N_3; B: the structure of partial MIF-GFP expression vector, pEGFP-MIF. MCS, EGFP, and MIF are the multiple cloning sites, enhanced green fluorescence protein gene and macrophage migration inhibition factor gene, respectively. The locations of the ATG, EGFP and MIF are indicated.

medium plates containing 50 μg/mL ampicillin. The plates were incubated at 37°C overnight. The positive clones were selected and cultured in the LB liquid medium containing 50 μg/mL ampicillin. The plasmid DNAs of the positive clones were extracted and identified by DNA sequencing. The sequencing primer, upstream of the inserted site, was 5′-CGGTAGGCGTGTACGGTG-3′.

2.4 Transfection of plasmid DNA into the HEK293 cell

The HEK293 cells were cultured in the DMEM medium with 10% fetal bovine serum at 37°C in humid air with 5% CO_2. Before the start of the experiment, 3×10^5 HEK293 cells were inoculated in 6-well plates. After the cell abundance reached 60% to 70%, the plasmid DNAs were transfected into the cells by using Trans IT-293 reagent. Nine groups were set, with 4 parallel wells of cells in each group. Among groups 1 to 5, the MIF-GFP expression plasmid was co-transfected into the HEK293 cells with MIF siRNA1, MIF siRNA2, MIF siRNA3, negative siRNA and GFP siRNA as positive siRNA, respectively. Among groups 6 to 9, the MIF expression plasmid was co-transfected into the HEK293 cells with MIF siRNA1, MIF siRNA2, MIF siRNA3, and negative siRNA, respectively. The amount of DNA transfected into the cells in each well was 2 μg and the copies of siRNAs expression plasmid were equal to those of the *MIF-GFP* or *MIF* expression plasmid. Seventy-two h after transfection, the expressions of GFP in HEK293 cells from groups 1 to 5 were examined by fluorescent light microscopy. The total cellular RNA of each well was extracted by the Trizol reagent (Gibco-BRL, USA). The first-strand cDNAs were synthesized by using a mixture of oligo $(dT)_{12–18}$ primer with the superscript reverse transcriptase (Invitrogen, USA). Then, the expression of MIF mRNA in each group was detected by real-time quantitative PCR assay.

2.5 Real-time quantitative PCR assay

The plasmid DNA pSec-tag-*MIF* and T-GAPDH were used to establish the standard curves for the determination of MIF and GAPDH mRNA expression by real-time PCR assay. The real-time PCR was performed in the Quantitative PCR System (MJ Opticon II) using the following thermal cycling profile: 95°C for 1 min, followed by 40 cycles of amplification (94°C for 30 s, 58°C for 30 s, 72°C for 1 min). The absorption value of the SYBR Green I in each tube was detected at the end of each cycle. The melting curves analysis of the PCR products from 55°C to 95°C were also performed after the PCR amplification, followed by incubation at 12°C. The PCR was performed with the following primers: MIF forward primer, 5′-TCCATGACAACTTTGGTATCGT-3′, MIF reverse primer, 5′-CGTTGGCAGTGGGGACACG-3′ (228 bp); GAPDH forward primer, 5′-TTCATCGTAAACACCAACGTG-3′, GAPDH reverse primer, 5′-ACCCTGTCCGGGCTGATG-3′ (274 bp). The expression levels of the MIF mRNA were normalized to that of the GAPDH transcripts, which were measured using the same cDNAs. Reactions for each group were run in triplicate.

3 Results

3.1 Restriction and DNA sequencing identifications of the MIF-GFP fusion expression plasmid

Three positive clones were picked up from the LB solid medium ($Kana^+$) plates, and the plasmid DNA was extracted from the amplified clones in the LB liquid medium ($Kana^+$). The results of restriction enzyme digestion with *Hin*d III/*Bam*HI show that the DNA fragments with the same size as *MIF* gene were digested from 2 recombinant plasmids. DNA sequencing shows that the *MIF* gene was introduced into the p*EGFP-N*$_3$ vector with the right sequence and cloning sites in the 2 screened clones.

3.2 Screening for effective MIF siRNAs by using the MIF-GFP fusion expression plasmid

Seventy-two h after co-transfection of the p*EGFP-MIF* with the siRNAs expression plasmids into the HEK293 cells, the result of fluorescent light microscopy shows that compared with the negative control, the MIF-GFP was significantly inhibited in the *GFP* siRNA group (positive control) and was also silenced in other *MIF* siRNAs groups to different extent. Among the three *MIF* siRNAs, *MIF* siRNA1 could suppress the MIF-GFP expression more obviously (Fig. 2). The results of real-time PCR show that the *MIF* mRNA expression was consistent with the MIF-GFP expression after being interfered with the *MIF* siRNAs, and the *MIF* siRNA1 could inhibit the MIF mRNA expression most effectively with an inhibition rate of 66.67% (Fig. 3).

3.3 Screening for effective MIF siRNAs by using the MIF expression plasmid

The results of real-time PCR show that compared with the negative control, the *MIF* mRNA expression could be inhibited by the *MIF* siRNAs to different extents. Among the three *MIF* siRNAs, *MIF* siRNA1 could inhibit the MIF mRNA expression most effectively with an inhibition rate of 78.10% (Fig. 4).

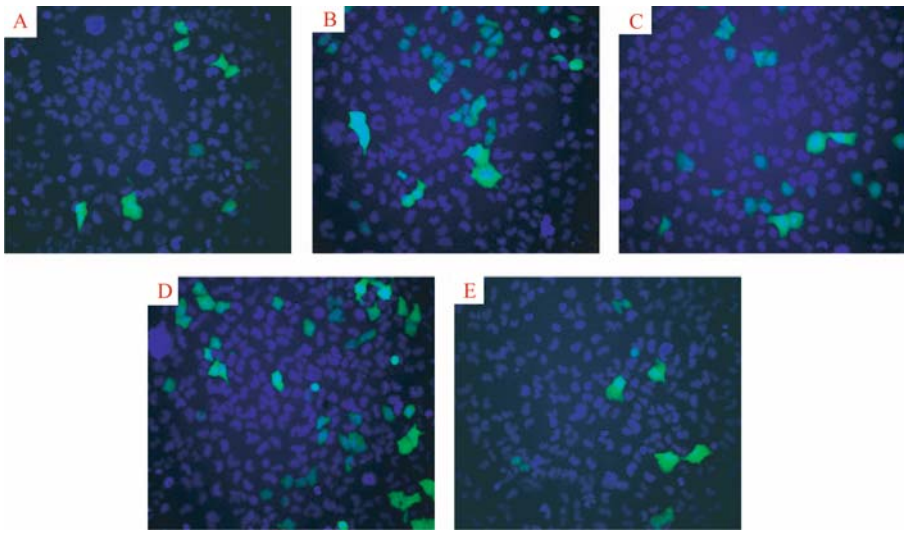

Fig. 2 Representative view of the MIF-GFP expression in the HEK293 cells. The pEGFP-MIF and siRNAs expression plasmids were co-transfected into the HEK293 cells by using the *Trans*IT-293 reagent. The fluorescence imaging of the MIF-GFP was measured as described in experimental procedures. A: HEK293 cells transfected with the pEGFP-MIF and MIF siRNA1 expression plasmid; B: HEK293 cells transfected with the pEGFP-MIF and MIF siRNA2 expression plasmid; C: HEK293 cells transfected with the pEGFP-MIF and MIF siRNA3 expression plasmid; D: HEK293 cells transfected with the pEGFP-MIF and pSilencer-Negative; E: HEK293 cells transfected with the pEGFP-MIF and GFP siRNA expression plasmid. The nucleoli of HEK293 cells were stained by the DAPI dye. Magnification × 200.

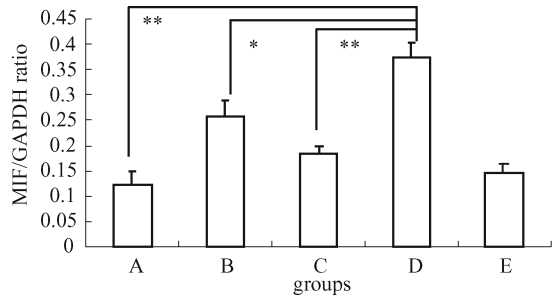

Fig. 3 Quantitative analysis of the MIF mRNA in the HEK293 cells transfected with the pEGFP-MIF and MIF siRNA expression plasmids by real-time PCR. Standard curves for the MIF and GAPDH were generated by serial dilution of each plasmid DNA. The expression levels of the MIF transcripts were normalized to that of the GAPDH transcripts, which were measured using the same cDNAs. Data were obtained from four experiments and are indicated as mean ± SD. A, B, C, D and E represent the corresponding groups in Figure 2. * : $P < 0.05$ and **: $P < 0.01$ *vs* negative control.

4 Discussion

Several methods could be used to prepare siRNAs to inhibit the expression of target genes, such as chemical synthesis of siRNAs in vitro, siRNAs prepared by in vitro transcription, double strand RNAs prepared by in vitro transcription degraded by the RNase III or Dicer enzymes to obtain a pool of siRNAs mix and shRNA expression vector or PCR product expression frame (Banan et al., 2004). The shRNA expression vector, also termed as siRNA expression vector, can be used to express shRNA in mammal cells. The expressed shRNAs will become siRNAs after the loop structure is digested by the Dicer enzyme. The siRNAs expression vectors include plasmid (Sui et al., 2002; Yu et al., 2003), adenovirus and lentivirus (Ogorelkova et al., 2006; Chen et al., 2006; Stewart et al., 2003; Morris et al., 2006). siRNAs mediated by expression vectors can initiate gene silencing for a long period or consistently and it is a commonly used method for RNAi study.

In this paper, the MIF siRNAs expression plasmids were transfected with the MIF-GFP fusion expression plasmid into the HEK293 cells to screen for effective siRNAs by using the GFP as a reporter. According to the sequence characters of the p*EGFP*-N3, there was a Kozak consensus translation initiation site and translation start code ATG in the EGFP open reading frame. If the *MIF* gene was inserted in the multi-cloning sites at the 5′ upstream of the EGFP ORF, the *MIF* would not co-express with the GFP as a fusion protein and the expression of the GFP will not be affected whether the MIF is expressed or not. Therefore, we constructed the MIF-GFP fusion expression vector, in which the *MIF* gene shared the same Kozak consensus translation initiation site and the ATG code with the *EGFP* gene within the same ORF and if the MIF mRNA was degraded, the MIF-GFP protein would fail to be expressed and then the GFP could be used as a reporter for the MIF mRNA expression in this system. In the HEK293 cells transfected with the *MIF* siRNAs and *MIF-GFP* expression plasmids, the *MIF* siRNAs can

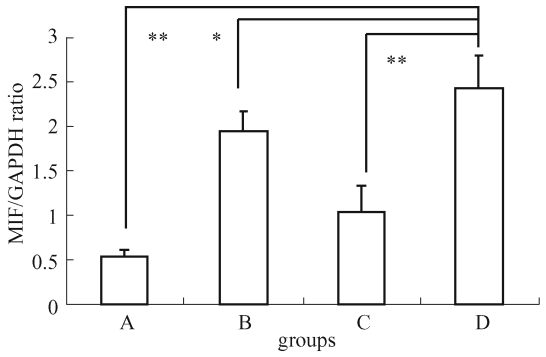

Fig. 4 Quantitative analysis of the MIF mRNA in the HEK293 cells transfected with the pSec-tag-MIF and MIF siRNA expression plasmids by real-time PCR. Standard curves for the MIF and GAPDH were generated by serial dilution of each plasmid DNA. The expression levels of the MIF transcripts were normalized to that of the GAPDH transcripts, which were measured using the same cDNAs. A: HEK293 cells transfected with the pSec-tag-MIF and MIF siRNA1 expression plasmid; B: HEK293 cells transfected with the pSec-tag-MIF and MIF siRNA2 expression plasmid; C: HEK293 cells transfected with the pSec-tag-MIF and MIF siRNA3 expression plasmid; D: HEK293 cells transfected with the pSec-tag-MIF and pSilencer-Negative. *: $P < 0.05$ and **: $P < 0.01$ *vs* negative control.

induce the degradation of *MIF* mRNA without the destruction of GFP mRNA at the 3′ downstream of the MIF-GFP transcripts. Therefore, the *GFP* mRNA level is not correlated with the levels of *MIF* mRNA and MIF-GFP, which was verified by the real-time PCR assay (data not shown). However, the results of the real-time PCR show that the *MIF* mRNA levels were consistent with the expression of GFP as a reporter as observed by using fluorescent light microscopy. On further studies, we detected the *MIF* mRNA expression in the HEK293 cells transfected with the *MIF* siRNA and *MIF* expression plasmids. The effective *MIF* siRNA was also identified, consistent with the screening result by using the GFP as a reporter. Together, the results of the 2 real-time PCR assays verified that the effective MIF siRNAs could be screened out by using the GFP as a reporter in the presently established system. In addition, the inhibitory degrees on the MIF mRNA expression induced by the *MIF* siRNA1 were different in the HEK293 cells transfected with the p*EGFP-MIF* or pSec-tag-*MIF*, which might be attributed to such factors as the transfection efficiencies and transcription levels of the two MIF expression vectors.

In conclusion, a useful method to screen for effective siRNAs by using the GFP as a reporter has been established. This simple and visualized method can be combined with histological immunochemistry and Western-blotting analysis with antibodies to target protein or GFP, and can also be combined with flow cytometry of the GFP expression to screen for effective siRNAs for the target genes. Moreover, this is also a good strategy to screen for effective siRNAs targeting multi-genes.

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References

Banan M, Puri N (2004). The ins and outs of RNAi in mammalian cells. Curr Pharm Biotechnol, 5(5): 441–450

Chen W, Liu M, Jiao Y, Yan W, Wei X, Chen J, Fei L, Liu Y, Zuo X, Yang F, Lu Y, Zheng Z (2006). Adenovirus-mediated RNA interference against foot-and-mouth disease virus infection both in vitro and in vivo. J Virol, 80(7): 3559–3566

Matzke M, Matzke A J, Kooter J M (2001). RNA: guiding gene silencing. Science, 293(5532): 1080–1083

Morris K V, Rossi J J (2006). Lentivirus-mediated RNA interference therapy for human immunodeficiency virus type 1 infection. Hum Gene The, 17(5): 479–486

Ogorelkova M, Zwaagstra J, Elahi S M, Dias C, Guilbaut C, Lo R, Collins C, Jaramillo M, Mullick A, O'Connor-McCourt M, Massie B (2006). Adenovirus-delivered antisense RNA and shRNA exhibit different silencing efficiencies for the endogenous transforming growth factor-beta (TGF-beta) type II receptor. Oligonucleotides, 16(1): 2–14

Stewart S A, Dykxhoorn D M, Palliser D, Mizuno H, Yu E Y, An D S, Sabatini D M, Chen I S, Hahn W C, Sharp P A, Weinberg R A, Novina C D (2003). Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA, 9(4): 493–501

Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester W C, Shi Y. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells (2002). Proc Natl Acad Sci USA, 99(8): 5515–5520

Yu J Y, Taylor J, DeRuiter S L, Vojtek A B, Turner D L (2003). Simultaneous inhibition of GSK3alpha and GSK3beta using hairpin siRNA expression vectors. Mol Ther, 7(2): 228–236