

Bipartite vectors for co-expression of a growth factor cDNA and short hairpin RNA against an apoptotic gene

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Abstract

Background Although human islet transplantation is a promising approach for treating type I diabetes, its success is limited as a result of the poor survival rate of transplanted islets. Expression of a growth factor gene to promote revascularization and silencing of pro-apoptotic genes before transplantation may improve the outcome of islet transplantation.

Methods In the present study, we constructed bipartite plasmid vectors to co-express a vascular endothelial growth factor (VEGF) cDNA and short hairpin (sh)RNA targeting inducible NO synthase (iNOS) gene. First, we screened shRNA sequences against human iNOS by transfecting plasmids encoding shRNA targeting different start sites of human iNOS. Then, the effect of different promoters [such as H1, U6 and cytomegalovirus (CMV)] and micro RNA backbones on gene silencing was determined.

Results No statistical difference in iNOS gene silencing was observed for the shRNA with H1, U6 and CMV promoters. In addition, a conventional shRNA showed better silencing of the iNOS gene compared to shRNA containing mir375 and mir30 backbones. A bipartite plasmid was also constructed with mir30-shRNA and a VEGF cDNA controlled by a single CMV promoter. This plasmid showed a better silencing effect compared to plasmid without VEGF cDNA.

Conclusions In the present study, we have successfully constructed bipartite vectors co-expressing a VEGF cDNA and a shRNA against the iNOS gene. These vectors could be attractive candidates for improving the survival of transplanted islets. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords iNOS; islet transplantation; RNAi vectors; shRNA; VEGF

Introduction

RNA interference (RNAi) is an evolutionarily conserved biologic process that regulates gene expression by small interfering double-stranded RNA (siRNA)-mediated sequence-specific, post-transcriptional gene silencing [1–3]. Several steps are involved in RNAi: (i) long double-stranded RNA is processed by Dicer into a 19–23 bp siRNA duplex; (ii) siRNA duplex is incorporated into a complex named RNA-induced silencing complex (RISC); and (iii) RISC is activated by eliminating the passenger strand of siRNA duplex, resulting in mRNA degradation or translational repression [4].

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RNAi has been extensively used as a tool for gene silencing in cell lines and animal models [5,6]. In addition, it is also a promising approach for treating various acquired and genetic diseases [7,8]. Recently, we have tested the effects of siRNAs against inducible nitric oxide synthase (iNOS) genes on insulin producing β -cell line (INS-1E) and on human islets [9,10]. iNOS gene silencing with chemically synthesized siRNA decreased NO production in INS-1E rat β -cells and human islets, reduced pro-inflammatory cytokine-induced β -cell death, and partially protected the human islet function [9]. In another study, we also observed that adenovirus-based short hairpin (sh)RNA against caspase-3 gene (Adv-caspase-3-shRNA) efficiently silenced caspase-3 gene, and its gene silencing effect lasted beyond 5 days, which resulted in the protection of islets from cytokine-induced apoptosis [11].

As an alternative cure for type I diabetes, human islet transplantation has made great progress, especially after the success of the Edmonton protocol [12,13]. However, a large number of patients returned to being insulin-dependent within 1 year after islet transplantation [14]. The main reason for the failure of islet transplantation is that less than 30% of the transplanted islets survive in the early days post-transplantation for several reasons, including (i) poor revascularization, which results in insufficient supply of oxygen and nutrition, and (ii) pro-inflammatory cytokines inducing islet β -cell death, including apoptosis and necrosis [15]. In our previous study, we investigated the use of bipartite plasmid or adenoviral vectors that co-express one gene for reducing the inflammatory response (e.g. hIL-1Ra) and another for facilitating revascularization [e.g. hepatocyte growth factor, vascular endothelial growth factor (VEGF)] [16,17]. This combinatorial approach showed a synergistic effect by working on two independent therapeutic targets. Therefore, it is possible to observe improved therapeutic efficacy with bipartite vectors that co-express VEGF cDNA and shRNA targeting an apoptotic gene such as iNOS or caspase-3.

To co-express two genes in a single vector, we can use two separate expression cassettes driven by two promoters or use the internal ribosome entry site (IRES) sequence [18,19]. The use of IRES is usually not preferred because the gene after IRES usually shows a lower expression level [20]. Co-expression of a shRNA and a gene with two separate promoters is similar to that of two different genes, but it might be different for co-expression of a shRNA and a gene with a single promoter. Unlike gene expression plasmid, which utilizes a RNA polymerase II (Pol II) promoter, shRNA expression can utilize both Pol II promoters [e.g. cytomegalovirus (CMV) promoter] and RNA polymerase III (Pol III) promoters (e.g. H1, U6 promoters). The IRES sequence is not necessary for co-expressing multiple shRNA or a combination of shRNA and cDNA within a single promoter [21–23]. It has also been reported that co-expression of shRNA and cDNA can be realized by inserting a promoterless shRNA within the intron of a gene [24].

A bipartite vector that can effectively silence a target gene and express a therapeutic gene properly is in great need for the treatment of various diseases, including diabetes, cancer and viral infection. siRNA targeting sequences and the type of promoters used will greatly influence gene silencing efficiency. A shRNA and cDNA could be expressed by two different promoters or by a single promoter. It was also reported that the micro (mi)RNA-based shRNA showed improved gene silencing compared to conventional shRNA [25,26]. In the present study, we systemically investigated these factors to identify a most optimal bipartite vector and to identify parameters defining a potent shRNA and gene co-expression vector.

Materials and methods

Materials

Fetal bovine serum (FBS) was purchased from Mediatech, Inc (Herndon, VA, USA). Penicillin/streptomycin, phosphate-buffered saline, 0.25% (w/v) trypsin-ethylenediamine tetraacetic acid and Dulbecco's modified Eagle's medium (DMEM) medium were purchased from Gibco-BRL (Gaithersburg, MD, USA). All oligonucleotides used for shRNA cloning were obtained from Integrated DNA Technology (Coralville, IA, USA). All the enzymes used in cloning were purchased from New England Biolabs (Ipswich, MA, USA).

Plasmids

Negative control shRNA plasmid and human iNOS cDNA plasmid were purchased from OriGene (Rockville, MD, USA). Plasmids encoding shRNA targeting five different regions of the iNOS gene were purchased from Open Biosystems (Huntsville, AL, USA). To generate pH1-shiNOS-CMV-GFP, p-U6-shiNOS, and pCMV-shiNOS, two shRNA oligonucleotides (synthesized by IDT DNA) were annealed and cloned into pRNAT-H1.1/shuttle (*Bam*HI, *Hind*III), pSIREN-Shuttle (*Bam*HI, *Eco*RI) and p-shuttle2(*Xba*I+*Afl*II), respectively. pH1-shiNOS-CMV-VEGF was generated by replacing the green fluorescent protein (GFP) gene with VEGF polymerase chain reaction (PCR) fragment from pCMV-VEGF₁₆₅. Briefly, pH1-shiNOS-CMV-GFP was digested with *Pfl*MI, followed by treating with Klenow enzyme. After purification, it was digested with *Nhe*I and then purified. VEGF gene was amplified by PCR using primers: forward (*Nhe*I): 5'-GCCTAGCTAGCTAGATGAACTTTCTGCTGCTTG-3'; reverse (*Dra*I): 5'-CGCTATTTAAATCACCGCCTCGGC TTGTCACATC-3'. To make pU6-shiNOS-CMV-VEGF, pSIREN-shuttle was digested with *I*-ceII and *Bam*HI, and the fragment with U6 promoter was sub-cloned into the *I*-Ceu and *Bam*HI site in pH1-shiNOS-CMV-VEGF. pU6-mir375-shiNOS was generated by cloning annealed mir375 oligonucleotides into pSIREN-shuttle

(*Bam*HI, *Eco*RI). To make pU6-mir30-shiNOS, the mir30-shiNOS sequence was synthesized and sub-cloned into pSIREN-Shuttle (*Bam*HI, *Eco*RI). mir30-shiNOS sequence: GGATCCGTGCTCGCTTCGGCAGCACATATAC-TAGTCTCGACTAGGGATAACAGGGTAATTGTTTGAATAG-GCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTG-GAAACACTTGCTGGGATTACTTCTTCAGGTTAACCCAA-CAGAAGGGCGGCCGCAAGGTATATTGCTGTTGACAGTG-AGCGCGTGTATTTAACTGCCTTGTGTAGTGAAGCCACA-GATGTACACAAGGCAGTTAAATACACATGCCTACTGCC-TCGTCTAGAAAAGGGGCTACTTTAGGAGCAATTATCTTG-TTTACTAAAACCTGAATACCTTGCTATCTCTTTGATACA-TTTTTTgaattc. To make pCMV-mir30-shiNOS, the mir30-shiNOS sequence was amplified by PCR using the primers: forward (*Dra*I): 5'-GTATTTAAAGGATCCGTGCTCGCTTC-GGC-3'; reverse (*Afl*II): 5'-CGCCTTAAGAATGTATCAAAG-AGATAGCA-3' and PCR product was cloned into p-shuttle2 (*Dra*I, *Afl*II) after restriction enzyme digestion. pCMV-VEGF-mir30-shiNOS was made by inserting the VEGF PCR fragment between the CMV promoter and mir30 shiNOS sequence in pCMV-mir30-shiNOS using *Nhe*I and *Dra*I sites. All the plasmids were purified by Promega mini-prep kit (Promega, Madison, WI, USA) and confirmed by DNA sequencing.

Cell culture and transfection

AD293 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. In transfection experiments, cells were seeded in a 48-well plate at a density of 20 000 cells/well, 24 h before transfection. Then, 0.05 µg of iNOS cDNA plasmid and 0.3 µg of shRNA plasmid were co-transfected into cells with Fugene HD transfection reagent (Roche Applied Science, Indianapolis, IN, USA).

Real-time RT-PCR

Human iNOS and VEGF expression were determined at the mRNA level by real time RT-PCR. After treatment, total RNA was extracted with the RNeasy Mini kit and treated with DNase by on-column digestion (RNase-Free DNase Set) (Qiagen, Valencia, CA, USA). RNA concentration was determined by a spectrophotometer (NanoDrop; Thermo Fisher Scientific Inc., Waltham, MA, USA). One hundred and seventy nanograms total RNA was converted into cDNA using multiscribe reverse transcriptase reagents and random hexamers in a 10-µl reaction system. Two microliters of cDNA were used as a template and analysed by SYBR Green-I dye universal PCR master mix on a LightCycler 480 Instrument (Roche Applied Science). The primers used for real-time PCR were: human iNOS: forward 5'-ACGTGCGTTACTCCACCAACA-3'; reverse 5'-CATAGCGGATGAGCTGAGCA-3' (amplicon size 102 bp); human ribosomal protein S19 (human S19); forward 5'-GCTTGCTCCCTACCGATGAGA-3'; reverse 5'-ACCCCGGAGGTACAGGTG-3' (amplicon size 73 bp) [9].

The primer for VEGF gene was the same as that previously described [16]. To assess the specificity of the amplified PCR product, melting curve analysis was performed on a LightCycler 480 Instrument. The results at the iNOS mRNA level were compared by calculating the crossing point value and normalized by the reference genes (human S19).

Determination of NO production

NO is rapidly oxidized in culture medium into nitrite, which accumulates in the sample and can be easily correlated with NO production. Therefore, nitrite concentration was determined using the Griess assay (Promega). Fifty microliters of cell culture supernatant was added to a 96 well-plate and mixed with 50 µl of 1% sulfanilamide in 5% phosphoric acid solution and incubated for 5 min at room temperature in the dark. Then, 50 µl of 0.1% *N*-1-naphthylethylenediamine dihydrochloride aqueous solution was added to each well. The plate was incubated for an additional 10 min, and absorbance was measured at 560 nm using a microplate reader. To determine the nitrite concentration in each sample, a standard curve was prepared using nitrite standard solution and culture medium as matrix.

Determination of VEGF expression from bipartite plasmids with enzyme-linked immunosorbent assay (ELISA)

At 36 h after transfection of AD293 cells with 0.3 µg/well plasmids, culture medium of AD293 cells was collected. The VEGF concentration in the culture medium was measured by ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

Results

Effect of targeting sequence on iNOS gene silencing

Targeting sequence is one of the most important elements for effective gene silencing. Therefore, we first screened five plasmids encoding shRNA targeting different regions of human iNOS gene. The targeting sequences of different plasmids are shown in Figure 1A. To find the most potent targeting sequence, plasmids expressing shRNA against five different iNOS mRNA regions were co-transfected with piNOS into AD293 cells. Plasmid with scrambled sequence was used as a control. At 24 h after transfection, NO production from iNOS gene expression was measured as nitrite by the Griess assay. As shown in Figure 1B, all five shRNA expression plasmids were able to silence iNOS expression and, among them, the 4060

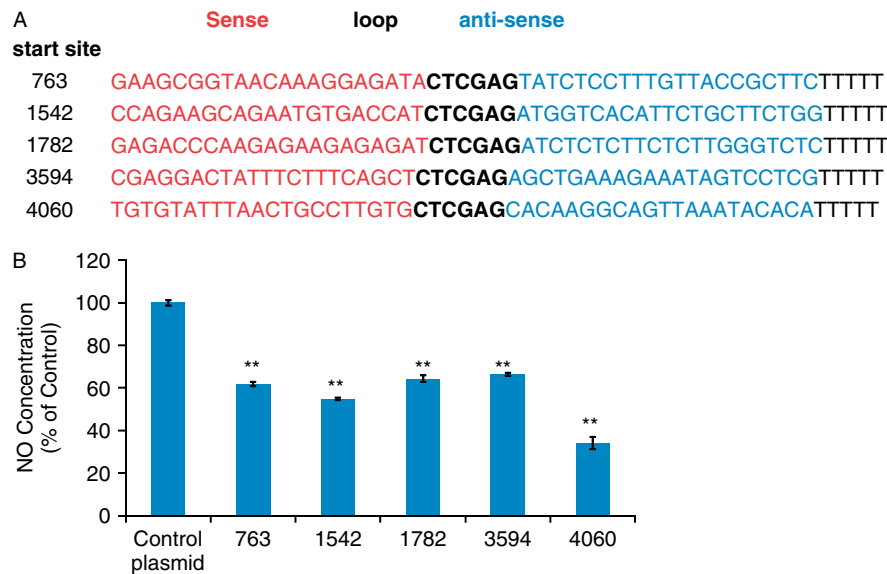


Figure 1. Effect of targeting sequence on iNOS gene silencing. (A) Sequences of shRNA against different regions of human iNOS gene (NM.000625). (B) Plasmids with different shRNA sequences, as well as control plasmid, were co-transfected with piNOS into AD293 cells. At 24 h after transfection, NO production was measured by the Griess assay. Nitrite concentration is expressed as a percentage of control. Results are the mean \pm SE ($n = 4$). ** $p < 0.01$ compared to the control plasmid group

showed a 66% reduction of NO production ($p < 0.01$). Therefore, the targeting sequence 4060 was used in the subsequent experiments for shRNA or miRNA-based shRNA construction.

Effect of promoters on iNOS gene silencing

The promoter is an important regulatory element in gene expression. Among several promoters, H1, U6 and CMV promoters are the most widely used for shRNA-mediated gene silencing. Because some discrepancies have been reported with respect to the efficiency of these promoters [26,27], we compared the efficiency of these three promoters for shRNA-mediated iNOS gene silencing by constructing iNOS shRNA expression plasmids with H1, U6 and CMV promoters (Figure 2A). After co-transfection with piNOS plasmid into AD293 cells, we found that, compared to the control group, iNOS shRNA expression plasmids could effectively ($p < 0.01$) reduce the NO production by 69%, 62% and 60% for H1, U6 and CMV, respectively (Figure 2B). No statistical difference in iNOS gene silencing was observed among the shRNA expression vectors driven by these promoters. This suggests that there is no significant difference among these three promoters in iNOS gene silencing.

Effect of VEGF co-expression on iNOS gene silencing

Co-expression of shRNA and therapeutic proteins is a promising combinatorial RNAi strategy [28], which will have a synergistic effect by acting on different

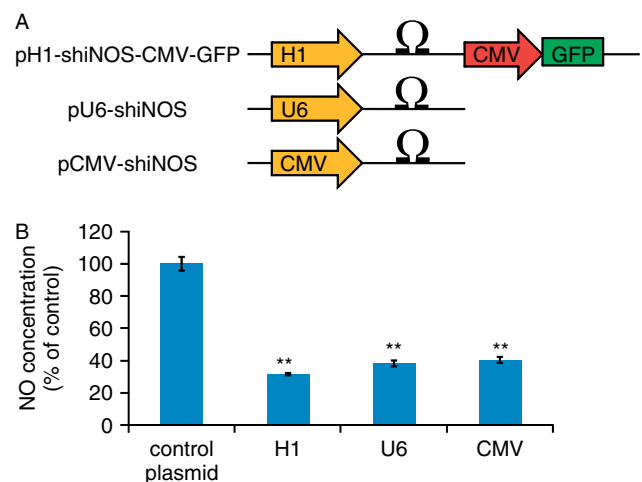


Figure 2. Effect of different promoters on iNOS gene silencing. (A) Structure of pH1-shiNOS-CMV-GFP, pU6-shiNOS and pCMV-shiNOS. (B) Plasmids with different promoters (H1, U6 and CMV), as well as the control plasmid, were co-transfected with piNOS into AD293 cells. At 24 h after transfection, NO production was measured by the Griess assay. Nitrite concentration is expressed as a percentage of control. Results are the mean \pm SE ($n = 4$). ** $p < 0.01$ compared to the control plasmid group

targets. However, it is not clear whether the over-expression of VEGF gene under a strong CMV promoter will interfere with the expression and processing of shRNA. As shown in Figure 3A, a bipartite plasmid has been constructed to co-express VEGF cDNA and iNOS-shRNA. VEGF cDNA was under the control of CMV promoter, whereas iNOS-shRNA was driven by U6 promoter. As shown in Figure 3B, compared to the control plasmid group, pU6-shiNOS-CMV-VEGF and pU6-shiNOS reduced NO production by 59% and 62% ($p < 0.01$), respectively. By contrast, the plasmid

expressing VEGF did not show a significant reduction of NO production. It was also noted that there was no significant difference between pU6-shiNOS and pU6-shiNOS-CMV-VEGF for NO production. Therefore, the co-expression of VEGF cDNA and iNOS-shRNA will not have much interference in the shRNA-mediated gene silencing.

Effect of mir375 and mir30 backbones on iNOS gene silencing

Efficient gene silencing has been reported when shRNA was embedded in a miRNA backbone [26,29]. To determine the possibility of increasing the iNOS gene silencing effect by shRNA with a miRNA backbone, two shRNA were designed based on mir375 and mir30 structures (Figure 4A). As shown in Figure 4C, significant reduction of NO production were observed in all of three vectors tested: pU6-shiNOS (reduced by 62%, $p < 0.01$), pU6-mir375-shiNOS (reduced by 39%, $p < 0.01$) and pU6-mir30-shiNOS (reduced by 30%, $p < 0.01$). In addition, pU6-shiNOS is significantly more efficient than pU6-mir375-shiNOS or pU6-mir30-shiNOS ($p < 0.01$). The gene silencing efficiency of these three shRNA vectors was also determined at the mRNA level by real-time PCR (Figure 4B). All of these three vectors reduced NO mRNA expression significantly ($p < 0.01$ compared to control). In addition, pU6-shiNOS (reduced by 56%) is significantly more efficient than pU6-mir375-shiNOS (reduced by 30%) or pU6-mir30-shiNOS (reduced by 28%) ($p < 0.05$, compared to pU6-shiNOS).

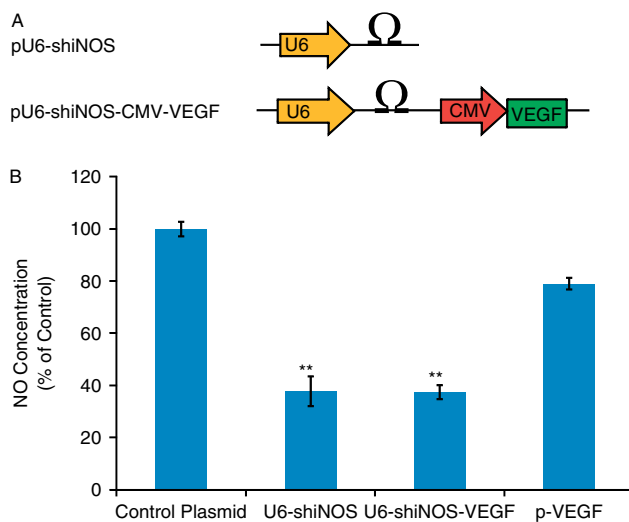


Figure 3. Effect of VEGF gene co-expression on iNOS gene silencing. (A) Structure of pU6-shiNOS, and pU6-shiNOS-CMV-VEGF. (B) Plasmids co-expressing shRNA and VEGF, plasmid expressing VEGF alone, as well as control plasmid, were co-transfected with piNOS into AD293 cells. At 24 h after transfection, NO production was measured by the Griess assay. Nitrite concentration is expressed as a percentage of control. Results are the mean \pm SE ($n = 4$). ** $p < 0.01$ compared to the control plasmid group

Insertion of the VEGF gene between the CMV promoter and mir30-shRNA enhanced iNOS gene silencing

We have shown the successful co-expression of iNOS shRNA and VEGF cDNA under two expression cassettes. To further investigate the possibility of co-expressing VEGF cDNA and iNOS shRNA under a single promoter, a plasmid with mir30-shiNOS under the control of a CMV promoter was constructed and a VEGF cDNA was inserted between the CMV promoter and mir30-shiNOS (Figure 5A). As shown in Figure 5B, a 39% reduction in NO production was achieved with pCMV-mir30-shiNOS ($p < 0.01$ compared to control plasmid), which is close to that of pU6-mir30-shiNOS (30%). However, the gene silencing effect was significantly enhanced by inserting a VEGF cDNA between the CMV promoter and mir30-shRNA. By contrast, a 61% reduction in NO production was achieved with p-CMV-VEGF-mir30-shiNOS ($p < 0.01$, with p-mir30-shiNOS).

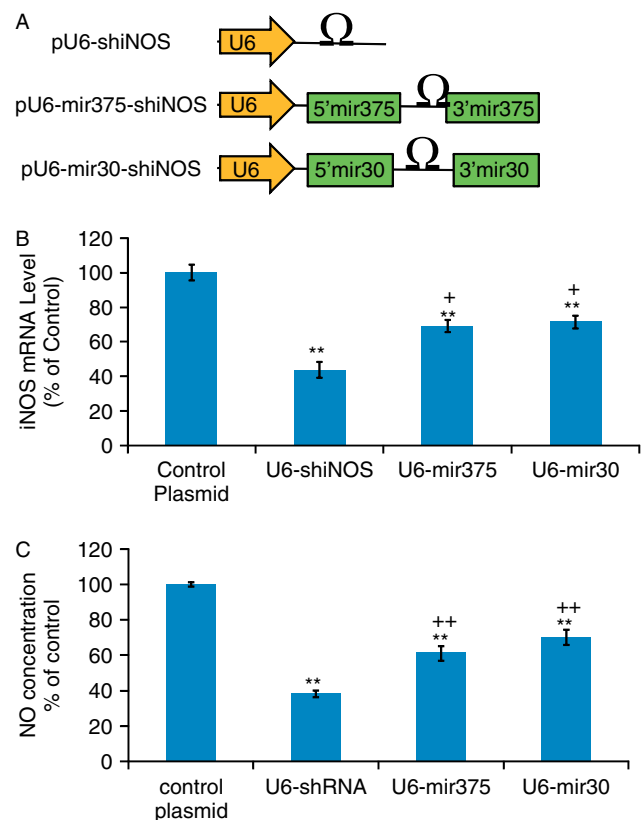


Figure 4. Effect of mir375 and mir30 backbone on iNOS gene silencing. (A) Structure of pU6-shiNOS, pU6-mir375-shiNOS and pU6-mir30-shiNOS. Plasmids with different backbones, as well as control plasmid, were co-transfected with piNOS into AD293 cells. At 24 h after transfection (B) iNOS mRNA levels was measured by real-time PCR (C) NO production was measured by the Griess assay. Nitrite concentration is expressed as a percentage of control. Results are the mean \pm SE ($n = 4$). ** $p < 0.01$ compared to the control plasmid group; ++ $p < 0.01$ and + $p < 0.05$, respectively, compared to the pU6-shiNOS group

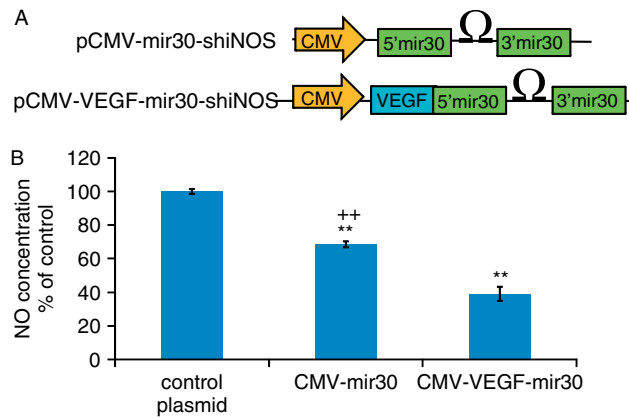


Figure 5. Insertion of the VEGF gene between the CMV promoter and mir30-shRNA enhanced iNOS gene silencing. (A) Structure of pCMV-mir30-shiNOS and pCMV-VEGF-mir30-shiNOS. (B) pCMV-mir30-shiNOS, pCMV-VEGF-mir30-shiNOS, as well as control plasmid, were co-transfected with piNOS into AD293 cells. At 24 h after transfection, NO production was measured by the Griess assay. Nitrite concentration is expressed as a percentage of control. Results are the mean \pm SE ($n = 4$). ** $p < 0.01$ compared to the control plasmid group; ++ $p < 0.01$ compared to the pCMV-VEGF-mir30 group

VEGF expression from bipartite plasmids

Not only potent silencing of the pro-apoptotic gene, but also sufficient VEGF expression is essential. Therefore, we measured VEGF gene expression from two bipartite plasmids, pU6-shiNOS-CMV-VEGF and pCMV-VEGF-mir30-shiNOS. We measured VEGF gene expression by ELISA of the cell culture medium. VEGF concentration was approximately 65 ng/ml for all above plasmids and there was no significant difference among these two plasmids (Figure 6B). VEGF gene expression from these two bipartite plasmids was also measured at the mRNA level with real-time PCR using VEGF gene specific primers (Figure 6A). The results obtained indicate that the VEGF mRNA levels in these two plasmid-treated groups were 914 ± 18 -fold and 922 ± 35 -fold higher than that in control group, respectively.

Discussion

The selection of potent targeting sequence that leads to effective gene silencing still remains a key issue for the practical application of the RNAi technique for disease therapy. Web-based computer programs with different algorithms for designing siRNA and shRNA are available. To increase the chance of finding a potent sequence, empirical rules are usually incorporated in these algorithms. These empirical rules include: thermodynamic property; length of siRNA target; GC content; and RNA secondary structure [30–32]. However, the selection of targeting sequence is still an empirical process and depends on the experimental screening of potential targets. This is because our understanding of RNAi

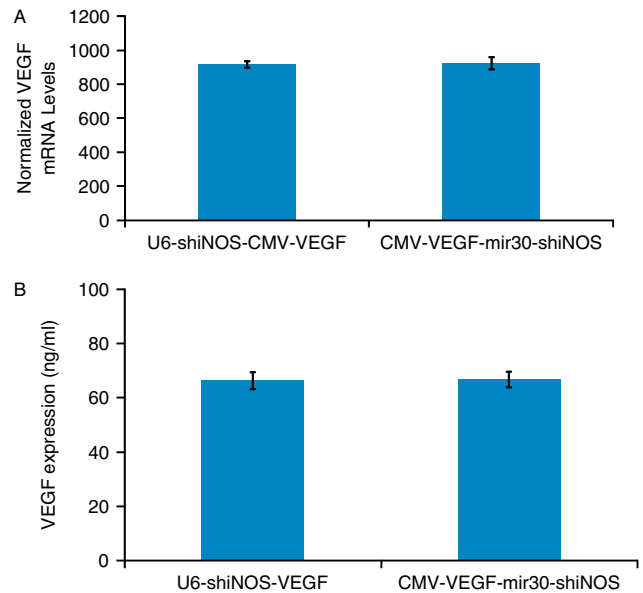


Figure 6. Expression of VEGF from pU6-shiNOS-CMV-VEGF and pCMV-VEGF-mir30-shiNOS. pU6-shiNOS-CMV-VEGF and pCMV-VEGF-mir30-shiNOS was transfected into AD293 cells. (A) At 24 h after transfection, VEGF gene expression levels were measured by real-time PCR (B) At 36 h after transfection, VEGF expression was measured by ELISA. Results are the mean \pm SE ($n = 4$)

mechanism is still insufficient. In addition, most of the algorithms for shRNA sequence design actually comprise that of siRNA design and convert the siRNA into the shRNA sequence. In the present study, we have converted a potent siRNA targeting sequence into a shRNA sequence with success [7,11]. However, we also observed a loss of the silencing effect after converting the potent siRNA sequence into shRNA (data not show). Therefore, a better understanding of the RNAi mechanism is required for improved the design of shRNA. In the present study, five shRNA expression plasmids were purchased and screened to determine the potent shRNA targeting sequence. In practice, this might be an efficient way to find a potent shRNA for certain applications.

The promoter is another important element that determines the duration, intensity and specificity of gene expression [33,34]. The activity of several promoters, including tRNA, H1, U6, CMV, LTR and CMV enhancer/H1, has been investigated [23,26,35]. In the present study, we did not observe a significant difference in the levels of iNOS gene silencing when CMV, U6 and H1 promoters were used for driving shRNA-iNOS. Similar results have been reported in a previous study in which it was demonstrated that, when a less efficient luciferase shRNA sequence was used, there were some differences in luciferase gene silencing among the different promoters; with the H1 promoter being less efficient than CMV and U6 promoters. However, when a potent luciferase shRNA sequence was used, the difference in gene silencing effect among the different promoters became minimal [26].

The use of miRNA-shRNA in siRNA expression vector is an attractive strategy, which mimics the structure of

natural occurring miRNA and thus can be processed by cellular miRNA machinery more efficiently compared to conventional shRNA [26]. Almost 80% more effective in reducing HIV p24 antigen production was achieved with TAT shRNA using miR-30 backbone compared to conventional shRNA [25]. shRNA is expressed in high level and yields an abundance of precursor, whereas miRNA-based shRNA is expressed at low levels but is processed more efficiently [36]. To further improve the silencing effect, we designed two shRNAs based on the mir375 and mir30 sequences. The gene silencing potency of mir375-shRNA and mir30-shRNA was compared with that of conventional shRNA. However, the shRNA showed the best gene silencing effect (i.e. 62% reduction of NO production), whereas mir375 or mir30 shRNA are less potent, with 39% and 30% reduction of NO production, respectively. Similar findings have also been reported in other studies [27,37]. Li *et al.* [27] compared conventional shRNA and miR30-based shRNAs against luciferase gene or mouse tyrosinase. Among the 14 different targeting sequences tested against luciferase, the conventional shRNA showed a better silencing effect than miR30-based shRNA in 11 sequences. All the conventional shRNA with ten different targeting sequences against mouse tyrosinase showed a significantly better silencing effect than the miR30-based shRNA counterpart. These authors explained that the shRNA structure used previously for comparison [26], which has a 29-nt stem and a 4-nt loop, is not an optimal design for shRNA. When a 4-nt loop sequence was used in shRNA, an insufficient processing of shRNA by Dicer was observed, which resulted in poor gene silencing [27]. However, when a 9-nt loop (UUCAAGAGA) and 19-nt stem structure was used in shRNA design, a better gene silencing was observed in conventional shRNA compared to mir30-based shRNA. Therefore, it is reasonable to observe a better gene silencing effect in the shRNA used in the present study, which has a 21-nt stem and 6-nt loop (CTCGAG). Boudreau *et al.* [37] also demonstrated that optimized shRNAs are more potent than mir30-based shRNAs for silencing of three genes, including GFP, SCA 1 and HD, when the variables were minimized in the comparison [37]. In the present study, the ultimate goal was to develop a vector for therapeutic purpose; thus, the conventional shRNA, which showed a better iNOS gene silencing effect, will be used in our future studies.

Another important feature of miRNA-based shRNA is that they are more amendable to Pol II transcription and polycistronic strategies, allowing delivery of multiple siRNA sequences (or a siRNA sequences and a cDNA) with a single promoter [21,22,38]. We first designed a miRNA30 based shRNA plasmid p-CMV-mir30-shiNOS, but it was not as potent as we expected. However, when we inserted a VEGF cDNA between the CMV promoter and the mir30-shiNOS sequence (p-CMV-VEGF-mir30-shiNOS), the gene silencing effect was significantly increased. This result is consistent with the findings of a study by Stegmeier *et al.* [29], who reported that the insertion of GFP, dsRED and Neo genes between the

CMV promoter and the mir30-shRNA cassette increased the knockdown of Rb gene. The exact reason for this is still unknown. Probably, a certain spacing between the CMV promoter and mir30-shRNA is necessary for efficient gene silencing. The p-CMV-VEGF-mir30-shiNOS could be another bipartite vector for therapeutic application, which co-expresses shRNA and a cDNA with a single promoter.

Co-transfection approach for evaluating gene silencing effect has been extensively reported [25,27,36,37], and has allowed researchers to measure the gene silencing effect with convenience and reliability. Therefore, a co-transfection of piNOS plasmid was used to determine the gene silencing effect of our newly-constructed vectors encoding shRNA targeting different start sites of iNOS, promoters and backbones. The use of a stably transfected cell line has also been reported [35]. A C6 cell stably expressing firefly luciferase gene has been used to study the gene silencing effect of shRNA vectors against firefly luciferase gene. However, this is not appropriate for iNOS gene silencing because iNOS is up-regulated only after stimulation with cytokines or other reagents. By using a co-transfection approach, the iNOS mRNA was expressed from piNOS plasmid after transfection. This could mimic the scenario of the cytokine-induced iNOS gene much better than iNOS stable expressing cell lines, in which the iNOS gene was constantly over-expressed. In addition, there are several limitations for the use of stable cell lines: (i) the establishment of a stable cell line usually takes a long time (at least several weeks); (ii) Stable cells need to be continuously treated with antibiotics such as neomycin, puromycin, which may have unwanted effects on cells; and (iii) only a gene that is not toxic or will not interfere with the vital cellular process could be used to make a stable cell line. Because of above mentioned reasons, a co-transfection method would be a good approach for the evaluation of the gene silencing effect during the development of gene silencing vectors.

The present study aimed to construct bipartite plasmids that could co-express VEGF gene for promoting revascularization and shRNA against the iNOS gene to reduce human islet β -cell death. This combinatorial strategy will help to improve the survival and function of islet grafts by promoting revascularization and inhibiting apoptosis [15]. The reason for using bipartite plasmids is that it will reduce the use of total plasmid backbone, thus minimizing the immunogenic effect caused by bacterial plasmids [17]. In addition, the bipartite plasmids were specially designed as a shuttle plasmid for constructing adenoviral vectors. Moreover, two restriction enzyme sites have been preserved flanking the shRNA sequence in our bipartite vector. This feature will allow us to replace the shRNA sequence with a new one through directional cloning. We could conveniently construct pU6-shCas3-CMV-VEGF by replacing the shRNA sequence in pU6-shiNOS-CMV-VEGF and caspase-3 gene silencing was observed (data not shown.).

In conclusion, we have constructed bipartite plasmids that co-express VEGF cDNA and shRNA against iNOS. VEGF cDNA and shRNA were driven by two different

promoters or by one single promoter. These plasmids are properly designed, which allows us to conveniently change the shRNA sequence. In addition, all of these plasmids could be used as a shuttle plasmid for producing adenoviral vectors.

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