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GENE DELIVERY

Efficient expression of vascular endothelial growth factor using minicircle DNA for angiogenic gene therapy

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Abstract

The application of plasmid DNA (pDNA)-based gene therapy is limited by its inefficient transgene expression. In this study, minicircle DNA was evaluated for efficient vascular endothelial growth factor (VEGF) expression in skeletal muscle cells. Production of minicircle DNA encoding VEGF was studied by a semi-quantitative electrophoresis method leading to optimized bacterial culture conditions and producing high purity (86.6%) minicircle DNA. The VEGF minicircle DNA under control of the SV40 promoter (pMini-SV-VEGF) showed an increased amount of VEGF mRNA and up to 8 times more VEGF expression than a conventional plasmid (pSV-VEGF) in two different skeletal muscle cell lines (C2C12 and L8). Minicircle DNA with different promoters, including the SV40, CMV and chicken β-actin, was tested for VEGF expression in a C2C12 skeletal muscle cell line. The high VEGF expression generated by minicircle DNA stimulated efficient endothelial cell growth *in vitro*. Furthermore, minicircle DNA expressed higher VEGF compared to conventional plasmid in the tibialis anterior (TA) muscle of mice. Taken together, the results suggest that minicircle DNA is an efficacious gene vector for angiogenic VEGF expression in skeletal muscle and may be useful for treating peripheral arterial disease (PAD).

Keywords: Minicircle DNA; VEGF; Skeletal muscle cell

1. Introduction

pDNA has served as the simplest gene vector for *in vivo* gene transfection applications. Advantages of using pDNA include low toxicity, ease of large scale production, no integration into the host genome and no contamination from helper virus. As a result, pDNA-based gene transfer has been studied for the treatment of numerous diseases such as cancer [1], cardiovascular [2] and skeletal muscle related disorders [3].

The clinical significance of peripheral arterial disease (PAD) is revealed by its high occurrence rate and limited therapeutic treatments. pDNA delivery-based therapeutic angiogenesis in skeletal muscle has proven to be an alternative approach for the treatment of PAD [4,5]. Expression of angiogenic VEGF using naked pDNA has been shown to enhance the recovery of local blood flow and improve clinical disease indexes by stimulating the proliferation of vascular endothelial cells [6]. However, clinical efficacy is limited by low transgene expression [7,8] leading to the need for the development of more efficient gene delivery/vector systems.

Two major approaches have been proposed to improve transgene expression. The first approach is to increase the efficiency of pDNA delivery. These methods include (1) chemical methods such as polymer-based carrier mediated pDNA delivery [9,10]; (2) physical methods such as electroporation [11,12] or ultrasound microbubble-mediated pDNA delivery [13]. The second approach is to optimize the transgene expression cassette by testing different transcription/translation regulatory elements such as promoter [14,15], enhancer [16,17] and 3′ untranslated region [18,19]. In addition to these approaches, minicircle DNA has been developed as a more efficient gene vector [20,21].

It has been discovered that unmethylated CpG motifs in pDNA can trigger the activation of immune response which

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decrease gene transfection efficiency [22–25]. Bacterial-originated sequences such as the origin of replication and the antibiotic resistance gene contain the most abundant unmethylated CpG dinucleotides in pDNA. Minicircle DNA is circular double-stranded DNA which contains merely the transgene expression cassette (including promoter, enhancer, gene cDNA and poly(A) signal) without bacterial-originated sequences. Minicircle DNA is low in immunogenicity due to its lower content of unmethylated CpG dinucleotides [26,27]. In addition, the smaller size of minicircle DNA may exhibit greater *in vivo* diffusivity and availability compared to the conventional plasmid with the same transgene expression cassette. CpG motif-depleted plasmids, including minicircle DNA, have been studied for production of several therapeutic proteins, for example, adiponectin [28] and human factor IX [26,29]. The target organs for expressing these therapeutic proteins are liver and lung. To date, minicircle DNA has not been evaluated for the expression of VEGF in skeletal muscle.

In order to elucidate the limiting step for obtaining high purity minicircle DNA encoding VEGF, the production process of minicircle DNA in bacterial host cells (TOP10) was studied. *In vitro* transfection efficiency of minicircle DNA was compared to their respective conventional plasmids. Different promoters, including the SV40, CMV and chicken β-actin were evaluated for VEGF expression *in vitro* and *in vivo*. The enhanced proliferation of endothelial cells by higher VEGF expression from minicircle DNA was also observed *in vitro*.

2. Materials and methods

2.1. Materials

Growth medium (DMEM, medium 199 and F-12k), serum (fetal bovine serum (FBS) and horse serum) and trypsin/EDTA were obtained from Invitrogen (Carlsbad, CA). Chicken embryo extract was purchased from Accurate Chemical (Westbury, NY). Branched PEI (average molecular weight of 25 kDa, average degree of polymerization 580), endothelial cell growth supplement (ECGS), heparin and L-arabinose were purchased from Sigma Aldrich (St. Louis, MO). pCMV-VEGF was constructed as previously described [9]. Luria-Bertani (LB) and Terrific broth (TB) were purchased from Fisher Scientific (Pittsburgh, PA). pGL3-basic plasmid was purchased from Promega (Madison, WI). pβ-VEGF was constructed by inserting VEGF165 cDNA (acquired from pSV-VEGF) into pβ-Null plasmid [30] using the HindIII and XbaI restriction sites.

2.2. Precursor plasmid construction

Precursor plasmids including p2BADφC31-SV-VEGF, p2BADφC31-CMV-VEGF and p2BADφC31-β-VEGF (Fig. 1A) were constructed for producing minicircle DNA. p2BADφC31 plasmid was a generous gift from Dr. Mark A. Kay at Stanford University. To construct p2BADφC31-SV-VEGF, VEGF cDNA with the SV40 promoter was excised from pSV-VEGF by digesting at BglII and BamHI sites, then treated with Klenow fragment for blunt-end generation. p2BADφC31 was digested with SpeI and treated with Klenow fragment as well. The blunted SV40 promoter-VEGF expression cassette was ligated to blunted linear p2BADφC31 at room temperature. Transformation was performed using DH10B competent cells (Invitrogen, Carlsbad, CA) and selected on an ampicillin (100 μg/mL) containing LB agar plate. Positive clones were verified by restriction enzyme digestion and analyzed using gel electrophoresis. The same above procedure was performed to obtain p2BADφC31-CMV-VEGF and p2BADφC31-β-VEGF.

2.3. Induction and purification of minicircle DNA

The procedure for the homologous recombination reaction and the purification of minicircle DNA developed by Z.Y. Chen et al. [29] were followed with minor modifications. Five different protocols were designed to optimize the incubation conditions for producing minicircle DNA encoding VEGF.

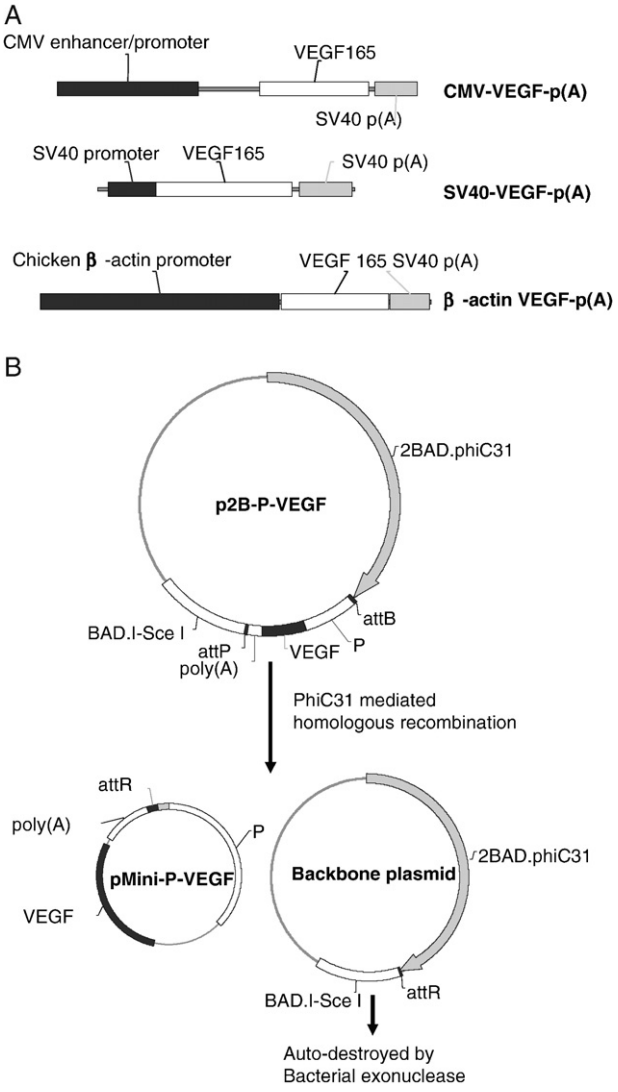

Fig. 1. (A) Structure of VEGF expression cassettes containing three different promoters including the CMV, SV40 and chicken β-actin promoters. (B) Schematic representation of the production process of minicircle DNA encoding a VEGF expression cassette. P: promoter; p2B-P-VEGF: precursor plasmid; pMini-P-VEGF: minicircle DNA.

Protocols I, III and V were performed in duplicate. Step 1: a fresh single colony of *E. coli* TOP10 (Invitrogen, Carlsbad, CA) transformed with precursor plasmid was inoculated in designated medium (LB for Protocols I and II; TB for Protocols III, IV and V) and incubated at 37 °C/250 rpm overnight (16–18 h). Cell pellets were obtained by centrifuging at 2800 rpm for 20 min at 20 °C. Step 2: fresh LB medium containing 1% (w/v) L-arabinose was added at a designated volume ratio to overnight culture medium (1:1 for Protocols I and III; 1:4 for Protocols II and IV; 1:8 for Protocol V) to re-suspend the cell pellet. The intra-bacterial φC31 integrase-mediated recombination was promoted at 32 °C for 2 h at 250 rpm. SceI endonuclease activity was then optimized by increasing the culture temperature to 37 °C for an additional 2 h. Plasmid purification was performed using Qiagen Maxiprep (Qiagen, Valencia, CA). As a result of recombination, the precursor plasmid splits into two smaller plasmids: backbone plasmid and minicircle DNA (Fig. 1B). The SceI site containing backbone plasmid was linearized by the subsequent expressed SceI endonuclease and finally cleaned up by intra-cellular exonuclease. The size of minicircle DNA is reduced to at least half of its original size compared to the conventional counterpart. For example, the size of pMini-CMV-VEGF (2 kb) is decreased from a larger size of pCMV-VEGF (4.6 kb).

2.4. Calculation of recombination and digestion efficiency

Upon induction with 1% arabinose, a precursor plasmid splits into two smaller plasmids including minicircle DNA and backbone plasmid. The amount of precursor plasmid or backbone plasmid depends on the activities of ϕC31 recombinase and SceI endonuclease. Assuming minicircle DNA was only produced from the designated recombination reaction and not subjected to SceI digestion, the efficiency for each reaction can be calculated semi-quantitatively based on the amount of minicircle DNA using the following equations:

$$\text{Overall efficiency (OE) (\%)} = \frac{(\text{WPb} - \text{WPa})}{\text{WPb}} * 100\%$$

$$\text{SceI digestion efficiency (SE) (\%)} = \frac{(\text{TBM} - \text{ABM})}{\text{TBM}} * 100\%$$

$$\text{Recombination efficiency (\%)} = \frac{\text{OE}}{\text{SE}} * 100\%.$$

Where,

TBM: Theoretical weight ratio of backbone plasmid/minicircle DNA
ABM: Actual weight ratio of backbone plasmid/minicircle DNA
WPb: Amount of precursor plasmid before recombination and SceI digestion
WPa: Amount of precursor plasmid after recombination and SceI digestion

The amount of each plasmid population can be quantified from gel electrophoresis analysis (Quanti-One, Bio-Rad, Hercules, CA). The molecular weight (MW) of minicircle DNA and backbone plasmid are required to calculate TBM. For example, MW of pBADφC31-CMV-VEGF is 12 kb; backbone plasmid is 10 kb, and MW of pMini-CMV-VEGF is 2 kb. Assuming the amount of minicircle DNA is *X*, then WPb is 6*X*. WPa can be calculated by dividing the amount of precursor plasmid by the amount of minicircle DNA.

2.5. Cell culture and in vitro transfection

Primary skeletal muscle (L8), mouse skeletal muscle (C2C12) and human umbilical vein endothelial cells (HUVEC) were purchased from ATCC (Rockville, MD). L8 cells were grown in culture flasks containing 10% horse serum, 5% chicken embryo extract and 20% medium 199 in DMEM medium. C2C12 cells were grown in culture flasks containing 10% fetal bovine serum (FBS) in DMEM medium. HUVEC cells were grown in culture flasks containing 10% FBS, 2 mM L-glutamine, 1.5 mg/mL sodium bicarbonate, 0.1 mg/mL heparin, and 0.04 mg/mL endothelial cell growth supplement (ECGS) in F-12 K medium. Every other day, the medium was replaced until cells became 80% confluent and then sub-cultured using 0.25% trypsin/EDTA treatment.

For the transfection assay, conventional plasmid or minicircle DNA was complexed with branched polyethylenimine (bPEI, 25 k) at a nitrogen/phosphate (N/P) ratio of 20:1. In order to maintain the same pDNA copy number for comparison, the amount of minicircle DNA was compensated by its molecular weight. For example, 0.43 μg pMini-CMV-VEGF (2 kb) was compared with 1 μg pCMV-VEGF (4.6 kb). pGL3-basic plasmid was used as an empty plasmid when complexing minicircle DNA with bPEI(25 k). Thirty minutes after complexation, bPEI(25 k)/pDNA complex was added to transfect cells seeded at a density of $3–5 \times 10^4$ cells/well in 24-wells plates. Prior to transfection, medium was replaced with serum free medium. Transfections for each group were performed in triplicate and allowed to incubate subsequently at 37 °C/5% CO_2 for 4 h. After this time, medium was replaced with 1 mL of serum containing medium.

2.6. RT-PCR

RT-PCR was performed as described previously [2]. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration of total RNA was measured at 260 nm. Reverse transcription was performed using AMV transcriptase (Promega, Madison, WI) to amplify the first cDNA strand from 2 μg of RNA. cDNA strands were further amplified with platinum *pfx* polymerase (Invitrogen, Carlsbad, CA). The RT-PCR of β-actin was also performed as an internal control. The sequences of primers were as follows: VEGF forward primer, 5′-CCC AAG CTT GAA ACC ATG AAC TTG CT-3′; VEGF backward primer, GCT CTA GAT CAT TCA TTC ACC GCC T-3′; β-actin forward primer, 5′-TGG AAT CCT GTG GCA TCC ATG AAA-3′; β-actin backward primer, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′. PCR reaction was: 45 °C for 45 min, 94 °C for 3 min, 20 cycles at 94 °C for 30 s, 58 °C for

30 s, and 72 °C for 1 min. The PCR products were separated by electrophoresis on a 0.8% agarose gel at 30 min.

2.7. In vitro endothelial cell proliferation assay

An *in vitro* endothelial cell proliferation assay was performed as described previously [2]. Medium (250 μl) was collected from C2C12 cells transfected with minicircle DNA or conventional plasmid, then added to ECGS-starved HUVECs (750 μl 10% FBS/DMEM) plated at a density of 2.5×10^4 cells/well in 24-well plates. After 5 days, proliferation of HUVECs was measured using a MTT assay. Briefly, 50 μl of 5 mg/mL MTT solution was added into each well and incubated for an additional 3 h. The medium was replaced by adding 100 μl of DMSO and the absorbance at a wavelength of 570 nm (OD_{570}) was measured using a microplate reader (Bio-Rad, Hercules, CA). HUVEC cells without any treatment were used as the relative control. The effect of VEGF on HUVEC proliferation is calculated by dividing the corrected absorbance of sample (OD_{570}(sample) − OD_{570}(control)) by the absorbance of control (OD_{570}(control)):

$$\text{Increased HUVEC cell proliferation (\%)} = \frac{OD_{570}(\text{sample}) - OD_{570}(\text{control})}{OD_{570}(\text{control})} \times 100\%.$$

2.8. In vivo transfection

Male Balb/c mice (approximate 15–20 g) were maintained in accordance with the guidance of the University of Utah Animal Care and Use Committee. Animals were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine through intra-peritoneal injection. An incision was made on the skin accessing the tiabialis anterior (TA) muscle. Minicircle DNA or conventional plasmid prepared in 30 μl of saline was injected into the TA muscle horizontally. After injection, the incision was sutured, and antibiotic cream was applied to prevent infection. Mice were euthanized by CO_2 inhalation two days after injection. TA muscles were completely excised, weighed and homogenized with an appropriate amount (2.5 μl/mg tissue) of reporter lysis buffer (Promega, Madison, WI) containing 2% protease inhibitor cocktail (Sigma, St. Louis, MO) in liquid nitrogen. The lysed mixtures were vortexed at room temperature for 30–40 min then centrifuged at 6000 g for 15 min. Supernatants were collected and preserved at −70 °C before further treatment.

2.9. VEGF ELISA

ELISA was performed using the Quantikine Human VEGF ELISA kit (R&D Systems, MN). Each sample (200 μl) was added into the designated wells. Similarly, VEGF standards ranging from 0.03 to 2 ng/ml were added to microwell plates. Calibrator diluent alone was added in blank wells and incubated at room temperature for 2 h. After washing three times with wash buffer, 200 μl polyclonal antibody against VEGF conjugated to horseradish peroxidase was added and incubated at room temperature for 2 h. After washing wells again three times, tetramethylbenzidine substrate solution was added and incubated at room temperature for 20 min. The enzymatic reaction was stopped by adding 50 μl of stop solution to each well, and absorbance was determined by spectrophotometric reading at 450 nm subtracting 540 nm. The VEGF concentration in samples was calculated based on a standard curve.

3. Results

3.1. Study of minicircle DNA production

pMini-CMV-VEGF was used for studying the process of minicircle DNA production. Minicircle DNA produced by using designated protocols was purified using Qiagen Maxiprep and analyzed by gel electrophoresis (Fig. 2). Weight purity of each plasmid population was analyzed and calculated using Quanti-One program (Bio-Rad, Hercules, CA). The initial use of LB broth (Protocol I) as overnight culture medium was found to give a moderate weight purity (53.8%) and molar purity (88.0%). To further improve this process, it is necessary to elucidate the production process of each plasmid population. To analyze the production process of minicircle DNA, equations were derived for calculating overall efficiency, φC31-mediated recombination efficiency and SceI-mediated digestion as described in the Materials and methods section. According to these equations, the φC31-mediated recombination efficiency was 90.7% and SceI-mediated digestion efficiency was 97.1% from using Protocol I (Table 1). By using TB (Protocol III), the purity of minicircle DNA was increased from 53.8% to 72.1% (Table 1). Since TB is an enriched culture medium and allows up to three times higher density of host cell growth, we tested three different volume ratios of 1% arabinose containing LB (1:1, 1:4 and 1:8) for re-suspending cell pellets in step 2. The purity of produced minicircle DNA was further improved from 72.1% to 86.6% by using a lower volume ratios (1:4 and 1:8) of re-suspended medium (Table 1).

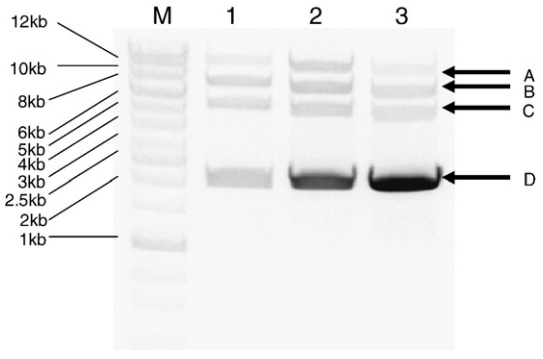

Fig. 2. Analysis of the production of minicircle DNA (pMini-CMV-VEGF) under different culture conditions (Protocols I, III and V). Minicircle DNA was purified as described in the Materials and methods. Purified DNA was digested with EcoRV and Spe1. After digestion, backbone plasmid was linearized (10 kb, arrow A), p2B-CMV-VEGF was cut into two linear fragments: (6.5 kb, arrow B) and (5.1 kb, arrow C), and mini-CMV-VEGF was linearized (2.1 kb, arrow D). M: DNA ladder (1 kb); 1: Protocol I; 2: Protocol III; 3: Protocol V.

Table 1
Summary of reaction efficiency for minicircle DNA production

		Batch number	Precursor plasmid (%)	Backbone plasmid (%)	Minicircle DNA (%)	PhiC31-mediated recombination efficiency (%)	Sce I-mediated digestion efficiency (%)	Overall efficiency (%)
Protocol I	LB 1:1	0116	39.9	7.6	52.5	89.9	97.1	87.3
		0503	36.9	8.0	55.1	91.5	97.1	88.8
Protocol II	LB 1:4	0503	29.9	7.8	62.3	94.3	97.5	92.0
Protocol III	TB 1:1	0116	21.1	8.4	70.5	97.3	97.6	95.0
		0503	17.4	8.8	73.7	98.4	97.6	96.1
Protocol IV	TB 1:4	0503	13.2	2.6	84.1	98.0	94.4	97.4
Protocol V	TB 1:8	0116	9.5	1.7	89.0	98.5	99.7	98.2
		0503	11.5	4.3	84.2	98.7	99.0	97.7

The amount of each plasmid population can be quantified from gel electrophoresis analysis (Quanti-One, Bio-Rad, Hercules, CA). The reaction efficiency was calculated using the equations in the Materials and methods.

3.2. Expression of VEGF protein by pMini-SV-VEGF in mouse and rat skeletal muscle cell line (C2C12 and L8 respectively)

The SV40 promoter has been used for transgene expression in skeletal muscle [31] and myocardial muscle [2]. bPEI (25 k) was used to deliver pMini-SV-VEGF and pSV-VEGF into skeletal muscle cell culture. The same copy number of each plasmid type was compared for VEGF production. The results (Fig. 3) show that VEGF expression from minicircle DNA was 2 to 8 times higher than conventional plasmid in mouse or rat skeletal muscle cell cultures, respectively.

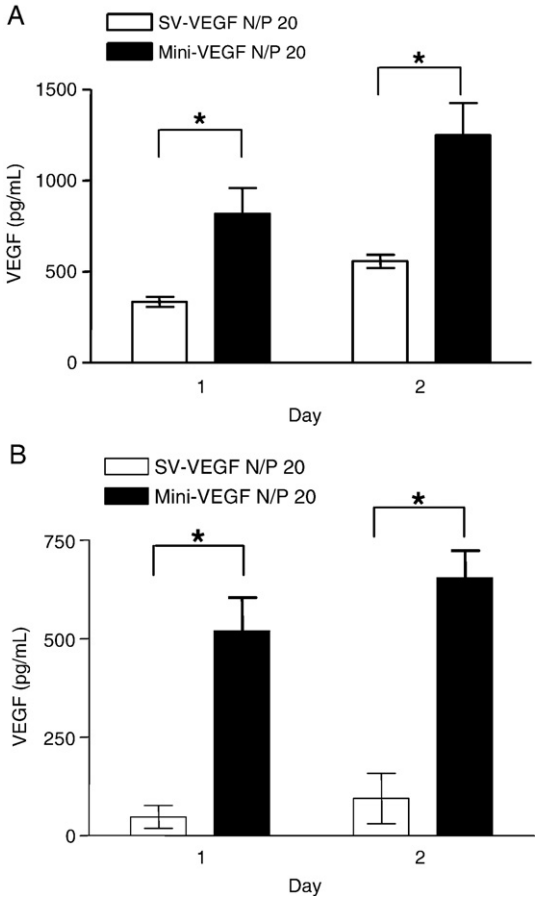

Fig. 3. VEGF gene expression after transfecting skeletal muscle cells with pMini-SV-VEGF or pSV-VEGF. The comparison was performed by using 1 μg pSV-VEGF or 0.25 μg pMini-SV-VEGF combining with 0.75 μg pGL3-basic (empty plasmid) on 4×10^4 cells per well. (A) L8 rat skeletal muscle cell. (B) C2C12 mouse skeletal muscle cell. Data represents the mean±SEM ($n=3$). Statistical analysis was done using Student's t-test. *$p<0.05$ compared to pSV-VEGF.

3.3. Expression of VEGF mRNA by different plasmid constructs in mouse skeletal muscle cell line (C2C12)

The higher transgene expression by minicircle DNA was also confirmed by comparing VEGF mRNA expression from transfected cells. RT-PCR was used as a specific and semi-quantitative method to evaluate the amount of VEGF mRNA expression. From the results in Fig. 4, amplified VEGF cDNA (~600 bp) from pMini-SV-VEGF transfected cells (lanes 1 and 2) was higher than pSV-VEGF transfected cells (lanes 3 and 4) and no VEGF cDNA could be detected from the control group (lanes 5 and 6).

3.4. VEGF expression from minicircle DNA with different promoters in skeletal muscle cell line

In this study, we compared VEGF expression by the SV40 promoter with two other commonly used promoters to obtain

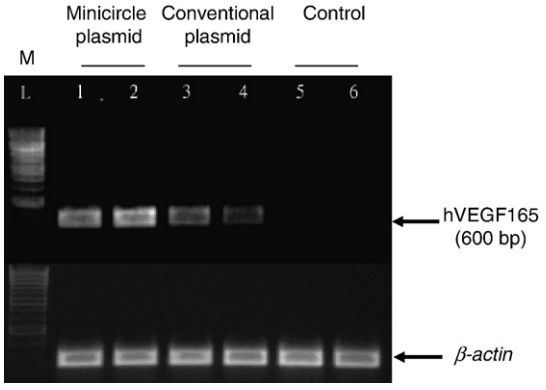

Fig. 4. Detection of VEGF mRNA expression after transfecting C2C12 cells with pMini-SV-VEGF or pSV-VEGF. Forty eight hours after transfection, total RNA was extracted and VEGF mRNA level was measured by RT-PCR. Detection of β-actin was used as the control.

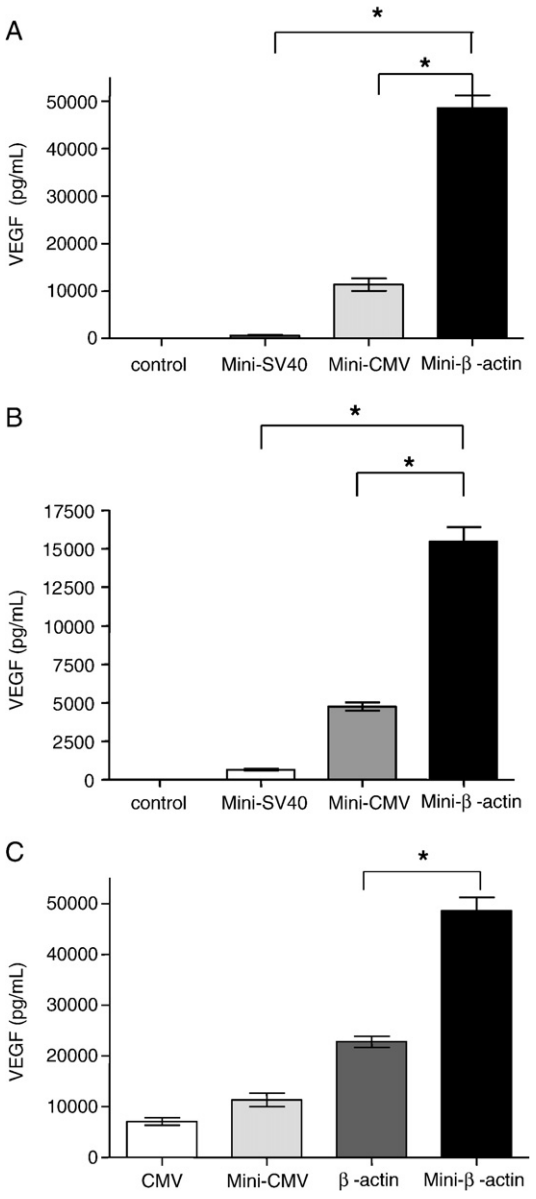

Fig. 5. VEGF protein expression after transfecting C2C12 cells with pMini-SV-VEGF, pMini-CMV-VEGF or pMini-β-VEGF. C2C12 cells without transfection were used as control group. (A) Forty eight hours after transfection. (B) Seventy two hours after transfection. (C) Comparison of VEGF protein expression between minicircle DNA and conventional plasmid under the CMV or chicken β-actin promoter. Data represents mean±SEM ($n=3$). Statistical analysis was done using one-way ANOVA and a Tukey's multiple comparison post-test. *$p<0.05$.

efficient VEGF expression from minicircle DNA in skeletal muscle cell line. The chicken β-actin promoter has been used to efficiently express GLP-1 [30] and adiponectin [28] *in vivo*. From the results (Fig. 5A, B), VEGF protein expression driven by the chicken β-actin promoter was higher than the CMV promoter and the SV40 promoter in C2C12 skeletal muscle cells 48 or 72 h after transfection. In addition, enhanced VEGF expression was observed from cells transfected by minicircle DNA with the chicken β-actin promoter or CMV promoter compared to their respective conventional plasmid (Fig. 5C).

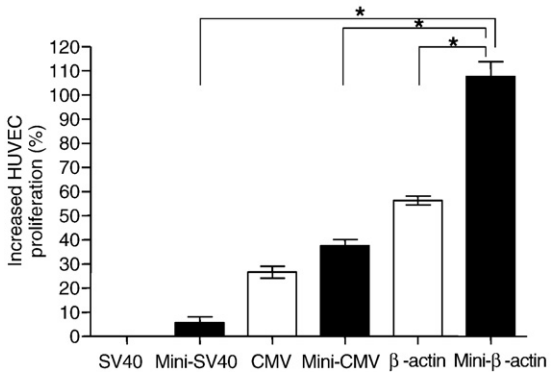

Fig. 6. *In vitro* endothelial cell proliferation assay. Forty eight hours after transfecting VEGF plasmid into C2C12 cells, the VEGF containing medium was added for HUVEC cell incubation. Five days after incubation, the cell population was measured by a MTT assay. Data represents mean±SEM ($n=3$). Statistical analysis was done using one-way ANOVA and a Tukey's multiple comparison post-test. *$p<0.05$ is considered as statistical difference.

3.5. In vitro endothelial cell proliferation assay

The advantage of achieving higher expression of VEGF by minicircle DNA was further investigated using an endothelial

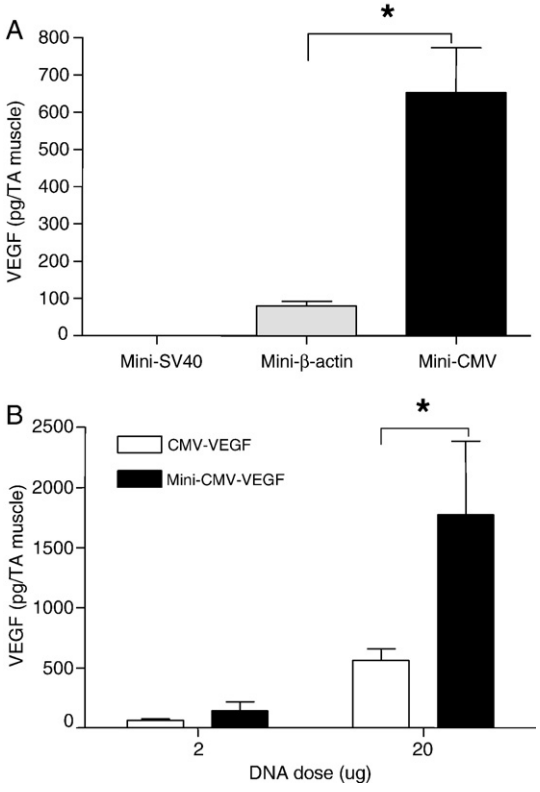

Fig. 7. *In vivo* transfection of minicircle DNA. Minicircle DNA or conventional plasmid was injected into TA muscle of mice. Two days after injection, the amount of VEGF in muscle was quantitated by ELISA. (A) Comparison of transcriptional activity of three different promoters: the SV40, chicken β-actin and CMV promoters in minicircle DNA. Ten μg of each minicircle DNA was injected into TA muscle for comparison. (B) Comparison of VEGF expression between minicircle DNA and conventional plasmid. Data represents mean± SEM ($n\geq 6$). Statistical analysis was done using Student *t*-test. *$p<0.05$ is considered as statistical difference.

cell proliferation assay (Fig. 6). Two days after muscle cells were transfected with VEGF pDNA, VEGF protein was secreted into 10% FBS/DMEM medium. The VEGF protein containing medium was collected and added to ECGS starving endothelial cells. According to our preliminary study, 10% FBS/DMEM does not enhance the proliferation of endothelial cells (data not shown). Therefore, the observed proliferation will be proportional to the amount of added VEGF protein. Five days after adding the VEGF protein containing medium from pMini-β-VEGF transfected cells, the number of endothelial cells was greater than cells receiving medium from pβ-VEGF transfected cells (Fig. 6).

3.6. In vivo transfection

Minicircle DNA or conventional plasmid was injected into TA muscle of mice. It has been shown previously that the peak time for VEGF expression is two days after injection [9]. In order to make an *in vivo* comparison, the expressed VEGF protein was extracted from muscle tissue two days after injection and quantified using ELISA. The order of VEGF expression by different promoters in minicircle DNA was: CMV > chicken β-actin > SV40 (Fig. 7A). Subsequently, minicircle DNA or conventional plasmid with the CMV promoter was compared for VEGF expression at two different doses (2 and 20 μg). Results (Fig. 7B) show that VEGF expression was three times higher with pMini-CMV-VEGF compared to conventional plasmid.

4. Discussion

Skeletal muscle is an immunogenic tissue [32] and is currently being studied as a gene vaccination target [25,33]. After injecting into skeletal muscle, unmethylated CpG motifs containing oligonucleotides or pDNA induces both immune [34] and inflammatory responses [35] accompanied with the secretion of IFN-γ which has been shown to strongly attenuate transgene expression from pDNA [36]. The use of minicircle DNA may reduce transgene silencing by eliciting lower immune responses compared to conventional plasmid. Also, the smaller size of minicircle DNA may contribute to a better extracellular and intra-cellular diffusivity [37] thus increasing its availability for transcription and translation processes.

The reaction efficiency for producing minicircle DNA from precursor plasmid depends on at least three factors including (1) size of expression cassette in minicircle DNA, (2) viability or activity of transformed host cells and (3) type of recombinant protein overexpressed in the host cells. First, the small size of VEGF expression cassette presents a significant problem for obtaining high weight purity of minicircle DNA. The short length of the VEGF expression cassette (1.1–2.2 kb in this study) between two recombination sites (attP and attB) may also affect the recombination efficiency. Second, assuming neither φC31 mediated recombination nor SceI-mediated digestion in dead host cells, a small decrease (i.e. 10%) of host cell viability or activity will result in a significant decrease on weight purity (40%). This is due to the low molecular weight of minicircle DNA (2 kb for pMini-CMV-VGF) compared to the larger molecular weight precursor (12 kb) or backbone plasmid (10 kb). For the effect of transgene product from pDNA in host cells, it has been shown that overexpression of recombinant proteins often results in serious metabolic changes and growth retardation [38]. All these factors discussed above may attribute to the production of minicircle DNA with low weight purity using Protocol I.

By using Protocol I, minicircle DNA with low weight purity (53.8%) and moderate molar purity (88.0%) was obtained. Assuming the precursor and backbone plasmid are equally susceptible to the degradation by SceI, a higher weight ratio (3:1) of precursor plasmid to backbone plasmid indicated that a significant portion of host cells in which precursor plasmids never initiated the recombination process. This speculation is also supported by obtaining minicircle DNA with higher SceI digestion efficiency (97.1%) and lower φC31 recombination efficiency (90.7%). We hypothesize that the decrease of host cell viability or activity is responsible for this observation. To increase the cell viability or activity, TB was used for overnight culture. TB has been discovered as an enriched medium for host cell (*E. coli*) growth to achieve better cell growth with higher plasmid yield. By using TB as the overnight culture medium, the recombination efficiency was increased from 90.7% to 98.6%. In TB, a favorable culture environment was provided for host cell growth and enzyme expression and activity. As a result, the weight purity of minicircle DNA was increased from 53.8% (Protocol I) to 86.6% (Protocol V).

For the *in vitro* transfection studies, bPEI(25 k) was used as a cationic carrier to enhance gene transfection efficiency in C2C12 cell culture. Higher expression of VEGF on both protein and mRNA levels were observed from minicircle DNA compared to conventional plasmids under different circumstances including different skeletal muscle cell lines and different days after transfection. The chicken β-actin promoter in minicircle DNA was found to show the highest VEGF expression among the promoters we tested *in vitro*. However, in skeletal muscle tissue, the CMV promoter showed higher VEGF expression compared to the chicken β-actin promoter. We speculate that the discrepancy of promoter comparison *in vitro* and *in vivo* might be due to the different genetic environment between immortal C2C12 myoblast culture and differentiated myotubes (muscle tissue). It has been reported that transcriptional activity of a promoter can be regulated by the differentiation status of muscle cells [39]. Our results support the concept that *in vivo* evaluation of promoter activity is crucial and may not be substituted by in vitro assay as suggested by other individuals [40].

VEGF-mediated therapeutic angiogenesis provides an effective alternative compared to conventional approaches such as surgical bypass and angioplasty. VEGF has been discovered as a survival and growth promotion factor for vascular endothelial cell proliferation under hypoxic conditions [41]. Histological chemistry studies have shown a correlation between increases of capillary density and recovery of blood perfusion [42,43]. In this study, HUVEC cells were used due to their resemblance to vascular endothelial cells [2]. By stimulation with higher VEGF expressed from minicircle DNA, we observed a dramatic

increase of HUVEC cell proliferation, which is an essential step for new capillary growth. It has been reported that VEGF expression in skeletal muscle can significantly stimulate the local blood perfusion. In this study, three times higher VEGF expression with minicircle DNA compared to conventional plasmid was observed in mouse skeletal muscle. The efficient *in vivo* VEGF expression from minicircle DNA holds great potential for the treatment of ischemia.

5. Conclusion

We have successfully cloned and purified minicircle DNA encoding the VEGF gene. Equations were derived to analyze the limiting step(s) for minicircle DNA production to optimize the production of minicircle DNA encoding VEGF expression cassettes specifically. The superior VEGF expression from minicircle DNA was demonstrated at the level of both transcription and translation levels in skeletal muscle cells. The potential of using minicircle DNA to stimulate vascular growth was evaluated on an *in vitro* endothelial cell proliferation model. The greater expression of VEGF from minicircle DNA resulted in a greater proliferation of endothelial cells. Furthermore, higher VEGF expression with minicircle DNA compared to conventional plasmid was observed in mouse skeletal muscle. To conclude, minicircle DNA is a potentially useful gene vector for therapeutic angiogenesis-based gene therapy.

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