Gene Transfer to Mouse Heart and Skeletal Muscles Using a Minicircle Expressing Human Vascular Endothelial Growth Factor

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Background: Gene transfer to heart muscle is a promising modality to treat ischemic heart disease. However, current vectors are inefficient and need to be improved. A novel vector system that shows great promise is the minicircle (MC) vector being smaller than conventional plasmid vectors and devoid of bacterial sequences.

Aims: To study gene transfer of MC DNA, expressing the human vascular endothelial growth factor (*hVEGF*), to mouse heart and skeletal muscles and to compare it with one of the efficient plasmids used in cardiovascular trials, the phVEGF165 containing the same expression cassette as the MC.

Results: The MC and the phVEGF165 plasmid show similar expression patterns both *in vitro* and in mouse heart and skeletal muscle studies *in vivo* on molar basis (equal expression in heart 24 hours, 0.9 fold lower expression from MC in heart and 1.9 fold higher in skeletal muscle at 7 days), whereas on weight basis the MC construct was more efficient in skeletal muscle (5.6 fold higher expression, $P < 0.05$), and at least as efficient in heart (1.6 fold higher expression).

Conclusions: The gene expression is similar in the 2 vector systems, so the smaller size and the fact that the MC construct is devoid of bacterial sequences and antibiotics resistance gene make the MC vector an attractive alternative for nonviral gene therapy.

Key Words: plasmid gene expression, in vitro, in vivo, gene therapy, efficacy, minicircle

(*J Cardiovasc Pharmacol*™ 2009;53:18–23)

Received for publication September 8, 2008; accepted November 6, 2008.

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Supported by the Swedish Research Council, Heart and Lung Foundation, Swedish Governmental Agency for Innovation Systems, Karolinska Institute, Belvéns stiftelse, and US National Institute of Health and European Union Sixth Framework Programme grants, Synthe Gene Delivery LSHB-2005108716 and Euro-Pharmaco-Gene LSHB-CT-2007-037283.

The authors report no conflict of interest.

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INTRODUCTION

Gene transfer to cardiac muscle using naked DNA is well established and has previously been used to treat patients with ischemic heart disease.[1] The results from these studies show that gene transfer is inefficient and the expression short-lived, this indicates that there is a need to improve the current vector systems. This can be done either by improving the gene transfer technique or by optimizing the plasmid construct with regard to elements such as the vector backbone, promoter, and polyadenylation (polyA) sites. For example, it has been suggested that the bacterial backbone negatively influences gene expression and contributes to the attenuation of gene expression in vivo.[2]

A possible solution to this problem is a recently presented minicircle (MC) system that is devoid of bacterial sequences as origin of replication and antibiotic resistance genes.[3–6] In this communication, we compare the expression of human vascular endothelial growth factor (*hVEGF*) from 2 plasmids with different backbones, the clinically used phVEGF165[7] and the commercially available pVAX1 (Invitrogen, Carlsbad, CA, USA). The highest expressing plasmid, phVEGF165.SR, was subsequently used to compare the efficiency of *hVEGF* expression from plasmid and MC constructs. We investigate the gene expression in vitro and in skeletal and cardiac muscles in vivo. To our knowledge, this is the first study where gene transfer of an MC construct into the heart muscle is performed.

METHODS

The Animal Care Committee of Karolinska University Hospital or the Local Ethical Committee at Karolinska Institute, Stockholm, approved all animal procedures.

Plasmid DNA

phVEGF165

The plasmid, phVEGF165 (Fig. 1A), contains an expression cassette for *hVEGF* with the human cytomegalovirus promoter and a rabbit β-globin polyA. It also contains a translation initiation signal and an untranslated herpes simplex virus thymidine kinase sequence upstream of the VEGF-A165 gene. The selection marker is a kanamycin (Km) resistance gene. The plasmid size is 6.6 kilobase pairs (kb), and the plasmid contains 0.05 CpG motifs per base pair. It was a generous gift of the late Dr Jeffrey Isner at St. Elizabeth's Medical Center, Boston, MA, USA.

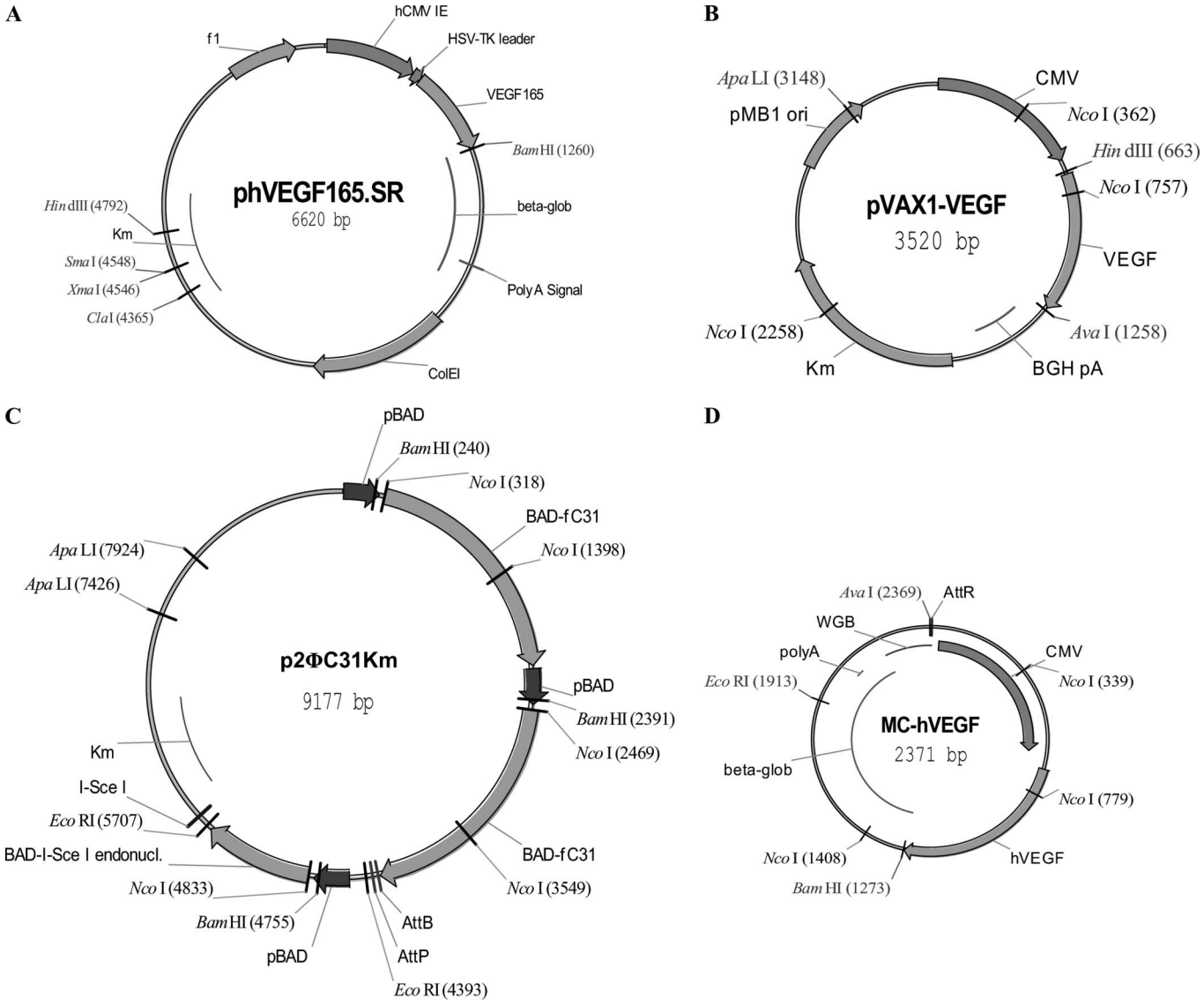

FIGURE 1. Plasmids used in study. A, phVEGF165 is a eukaryotic expression vector encoding the *hVEGF* isoform of 165 amino acids, transcriptionally regulated by a cytomegalovirus promoter/enhancer. B, pVAX1-VEGF is a plasmid based on the commercially available pVAX1 backbone. C, Schematic representation of the modified p2ΦC31 plasmid. The gene of interest is to be cloned between the AttB and AttP sites. D, MC-hVEGF: MC resulting from recombination of p2ΦC31-hVEGF carrying the same *hVEGF* cassette as phVEGF165. VEGF, vascular endothelial growth factor.

pVAX1-VEGF

The VEGF-A165 gene was cloned into the pVAX1 plasmid (Invitrogen) between the cytomegalovirus promoter and a bovine growth hormone polyA sequence. In pVAX1, eukaryotic DNA sequences are limited to those required for expression to minimize the possibility of chromosomal integration. Also, in this construct, the selection marker is Km and it contains 0.06 CpG motifs per base pair; the resulting plasmid pVAX1-VEGF is 3.52 kb (Fig. 1B).

The MC Construct

The entire expression cassette from phVEGF165 was cloned into the p2ΦC31 plasmid.[6] The p2ΦC31 plasmid seen in Figure 1C contains the gene for a restriction endonuclease, I-*Sce*I, and for its very rare recognition site. This gene is controlled by the arabinose inducible BAD-promoter, as are the 2 copies of the ΦC31 integrase gene. The gene for selection was replaced with Km resistance because ampicillin has been reported to cause an allergic response in sensitive individuals. The plasmid size is 9.3 kb, and with the *hVEGF* gene inserted, the resulting construct is 11.5 kb, with 0.08 CpG motifs per base pair. The 2.8-kb MC carrying the *hVEGF* gene expression cassette is produced through recombination at the Att sites (Fig. 1D); it contains 0.03 CpG motifs per base pair.

pGaussia

As control of transfection efficiency in the in vitro comparison of phVEGF and pVAX1-VEGF, a *Gaussia*

luciferase–expressing plasmid was used as internal reference, kindly provided by Iulian Oprea at the Karolinska Institutet, Sweden. *Gaussia* (hGLuc) is a humanized version of the luciferase gene from the marine copepod organism *Gaussia principes*, and the plasmid is described elsewhere.[8]

pCpG61lucS

As control for transfection efficiency in mouse skeletal muscle, we utilized pCpG61lucS, a completely CpG-free plasmid where the Luciferase gene is driven by the elongation factor I alpha promoter. It is 4.6 kb in size and has been generated by InvivoGene (San Diego, CA, USA).

Plasmid Production and Purification

The plasmids were prepared and purified from cultures of transformed *Escherichia coli* (*E. coli*) using a Qiagen mega kit (QIAGEN, GmbH, Hilden, Germany). The purity was checked with absorbance at 260 and 280 nm and by 1% agarose gel electrophoresis. The p2ΦC31-hVEGF was transformed by heat shock into *E. coli* strain One Shot Top10 (Invitrogen). Cells were grown overnight in terrific broth supplemented with 25 μg/mL Km, shaking with 225 revolutions per minute at 37°C. For induction, the pH of the culture was adjusted to 7.0 and L-(+)-arabinose was added to a final concentration of 1%. Recombination subsequently was allowed for 2 hours at 32°C. The I-*Sce*I endonuclease activity was then enhanced by adjustment of the pH to 8.0. The bacteria were harvested after 2 more hours of incubation at 32°C. The MCs were purified using a Qiagen mega kit (Qiagen GmbH). To remove remaining traces of unrecombined parental plasmid and bacterial backbone, the MC preparation was subsequently digested with *Afl*II, whose restriction site is present only in the bacterial backbone, followed by treatment with Plasmid-Safe adenosine triphosphate–dependent deoxyribonuclease (DNase) (Epicentre, Madison, WI, USA). The DNase-treated MCs were purified on a Qiagen PCR purification spin column (Qiagen GmbH).

Cell Lines and Transfection

The HT-1080 cell line was utilized for the in vitro comparison of the constructs. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 μg/mL gentamycin (DMEM 10). In the comparison of phVEGF165 and pVAX1-VEGF, 1 μg plasmid and 0.5 μg pGaussia were used in triplicates using FuGENE 6 (Roche, Mannheim, Germany). In the comparison of the MC construct, the cells were transfected in triplicates with 1 μg of phVEGF165 or equimolar proportions of MC or parental plasmid with FuGENE 6. All experiments were performed twice with a reagent:DNA ratio of 3:1. The cell cultures were harvested 48 hours after transfection.

Animals

Eight-week-old male C57BL/6 mice were used for the comparison of long-term gene expression of phVEGF165 and MC-hVEGF in mouse heart and skeletal muscles.

Intramyocardial Gene Transfer

The mice were anesthezised with 5 mg/kg midazolam, 0.1 mg/kg medetomidine, and 0.3 mg/kg fentanyl and subsequently tracheotomized. Ventilation was maintained using an Ugo Basile (Model 7025; Comerio, Varese, Italia). Through a left thoracotomy, the heart was exposed and injection was performed. Ten microliters of plasmid solution containing (1) 5 μg phVEGF165, (2) the equivalent molar amount of MC (1.85 μg), or (3) 5 μg MC in saline was injected into the free wall of the left ventricle of the heart.

Intramuscular Gene Transfer and Luciferase Activity Assay

The skeletal muscle injections were performed as described earlier[9]: 50 μL of saline solution containing 5 μg pCpG61lucS and (1) 5 μg phVEGF165, (2) the equivalent molar amount of MC (1.85 μg), or (3) 5 μg MC was injected into the *tibialis anterior* muscle (*sinister et dexterior*). Injection pressure and time were constant between the injections, and dorsal flexation of the talocrural joint was observed as a sign for correct injection. Live anesthetized mice were imaged for 15 seconds using an intensified charge-coupled device camera (IVIS Imaging System; Xenogen, MA) at 24-hour post injection.

Tissue Preparation

For the heart muscle, 6 mice treated with phVEGF165 or with the equivalent molar amount of MC-hVEGF were killed 24 hours after the gene transfer. After 7 days, 11 mice treated with phVEGF165, 8 mice treated with the same amount of MC-hVEGF, and 10 mice treated with the equivalent molar amount of MC-hVEGF were killed. For the skeletal muscle studies, 4 mice in all 3 groups were killed after 7 days. The muscle was extracted, rinsed in 1 × 24-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and lysed on ice in 200 μL of buffer containing 20 mM HEPES pH 7.4, 1.5 mM ethylenediaminetetraacetic, 0.5 mM phenylmethanesulphonylfluoride, 0.5 mM benzamidine, and 10 μg/mL trypsin inhibitor. The lysate was centrifuged at 25000*g*, for 10 minutes, at 4°C. The volume of the supernatant was adjusted to 500 μL and stored at −70°C until analysis.

Gene Expression Analysis

Expression of hVEGF165 was measured using an enzyme-linked immunosorbent assay for *hVEGF* (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol. The samples were analyzed in doublets and for the in vitro samples in 2 different dilutions. Heart muscle samples with an expression equal to the base level (1.5 ± 0.9 pg/heart) of *hVEGF* in hearts injected with saline only were considered unsuccessful injections and hence excluded. The hGLuc expression was quantified by fluorescence measurements on cell lysate after addition of coelenterazine (BioThema, Handen, Sweden).

Statistical Analysis

The data are presented as mean ± SEM. For the comparison of phVEGF165 and pVAX1-VEGF, the values were normalized by the hGLuc expression to compensate for differences in transfection efficiency. For the in vitro experiments, the *hVEGF* expression in each well was normalized against the mean expression for phVEGF165 in each group. The skeletal muscle values were normalized by the luciferase

activity at day 1 as a measurement of gene transfer efficiency. Statistical analysis was performed by Mann–Whitney *U* test and Student *t* test; a value of $P < 0.05$ was considered significant.

RESULTS

MC Production

The MC was produced in *E. coli* by a 1-step protocol. The bacterial degradation of unrecombined plasmid and MC was not complete, and in the preparations utilized, the MC proportion was calculated to be 70%–80% wt/wt through gel quantification. After degradation with Plasmid-Safe adenosine triphosphate–dependent DNase, no parental or bacterial backbone plasmid could be detected. In the cell culture expression analysis, it is evident that the expression from the parental plasmid is very low (Fig. 2B).

Comparison of *hVEGF* Expression From the Different Constructs In Vitro

To evaluate the plasmid efficacy in cell culture, HT-1080 cells were transfected with the *hVEGF* constructs. The phVEGF165 gave a much higher expression compared to a commercial plasmid construct carrying the *hVEGF* gene, pVAX1 (Fig. 2A). The MC and the phVEGF165 plasmid showed significantly higher gene expression than the parental plasmid (p2ΦC31-hVEGF). Furthermore, the MC yielded higher expression ($P < 0.05$) than phVEGF165 (Fig. 2B).

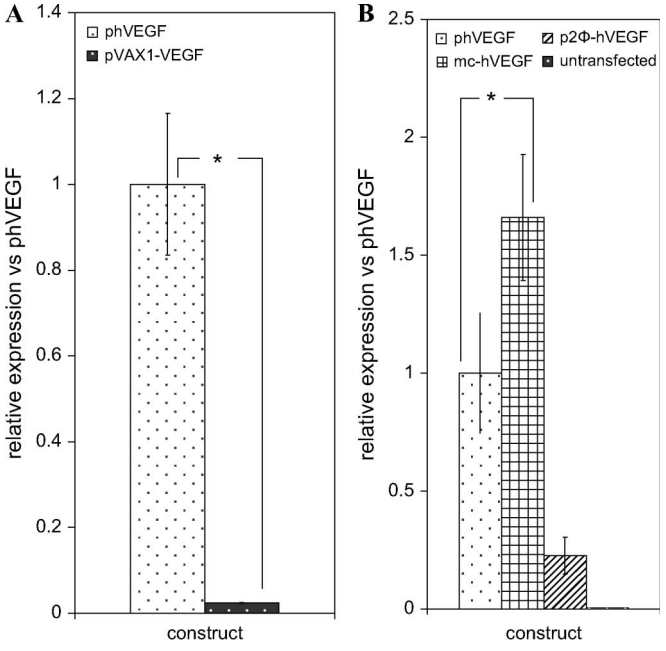

FIGURE 2. In vitro comparison between the different constructs. A, The graph shows relative VEGF concentrations normalized to the levels of expression from the cotransfected plasmid, pGLuc. The difference between phVEGF165 and pVAX1-VEGF is significant ($P < 0.05$) according to *U* test marked with an asterisk. B, The graph shows the relative VEGF expression. The *U* test shows that the difference between the MC and the phVEGF165 constructs is significant ($P < 0.05$) marked with an asterisk. All values are mean ± SEM. VEGF, vascular endothelial growth factor.

Long-term Expression In Vivo

Gene expression of the MC-hVEGF and the phVEGF165 in the mouse heart muscle and skeletal muscle was studied after gene transfer to the myocardium and the tibialis anterior muscle. After 24 hours, both constructs showed robust *hVEGF* gene expression in heart muscle (Fig. 3A). Interestingly, the gene expression remained significant at day 7 (Fig. 3B), although lower. The decrease over time was similar for the 2 constructs. Predictably, the *hVEGF* expression in the animals receiving the equimolar amounts of plasmid versus MC had a similar pattern, whereas the animals receiving the same dose in milligrams showed a higher expression for the MC samples. In skeletal muscle, the difference is statistically significant ($P < 0.05$, Fig. 3C). For the skeletal muscle samples, the MC showed a 5 times higher expression than the samples treated with the corresponding mass of plasmid. This can only partly be explained by the higher amount of expression cassettes. Furthermore, it is evident that the normalization of the *hVEGF* expression by the luciferase expression from a CpG-free plasmid with a different promoter is a robust way of compensating for differences in injection and gene transfer efficiency.

DISCUSSION

Gene therapy for ischemic heart disease shows promise, but clinical usage demands high security and stringent definition of the vector. Plasmid vectors are considered to be safer than the more commonly used viral vectors. However, the first-generation plasmid vectors contain bacterial sequences and selection markers such as genes conferring resistance to antibiotics. These sequences are thought to hamper gene expression and to trigger the immune system,[10] which might attenuate gene expression. Regulatory agencies in both the United States and the Europe recommend totally avoiding antibiotic resistance markers in DNA vectors to be used in clinical trials. In this article, we show that the MC can be transfected into and expressed in heart and skeletal muscles.[11,12] In the MC vectors, which may be considered the second-generation nonviral vectors, these sequences have been removed through recombination in the bacterial host during propagation; hence, the MC construct is a safer alternative. The gene expression from the MC-hVEGF is equal to that of a known high-expressing plasmid. Furthermore, on weight basis, the MC construct results in a greater expression, presumably due to the higher amount of expression cassettes. Hence, the same amount of DNA will give a higher molar dose when using the MC construct. In all, the MC construct reveals itself to be an interesting candidate for cardiovascular clinical trials.

In our study, we chose to compare the novel MC with the clinically used plasmid, phVEGF165.SR. This plasmid is outstanding in its capacity to express *hVEGF* in cell cultures as compared with a commercial minimal plasmid construct, the pVAX1-VEGF (Fig. 2A). The MC and the phVEGF165 plasmid show comparable expression patterns in vitro, in

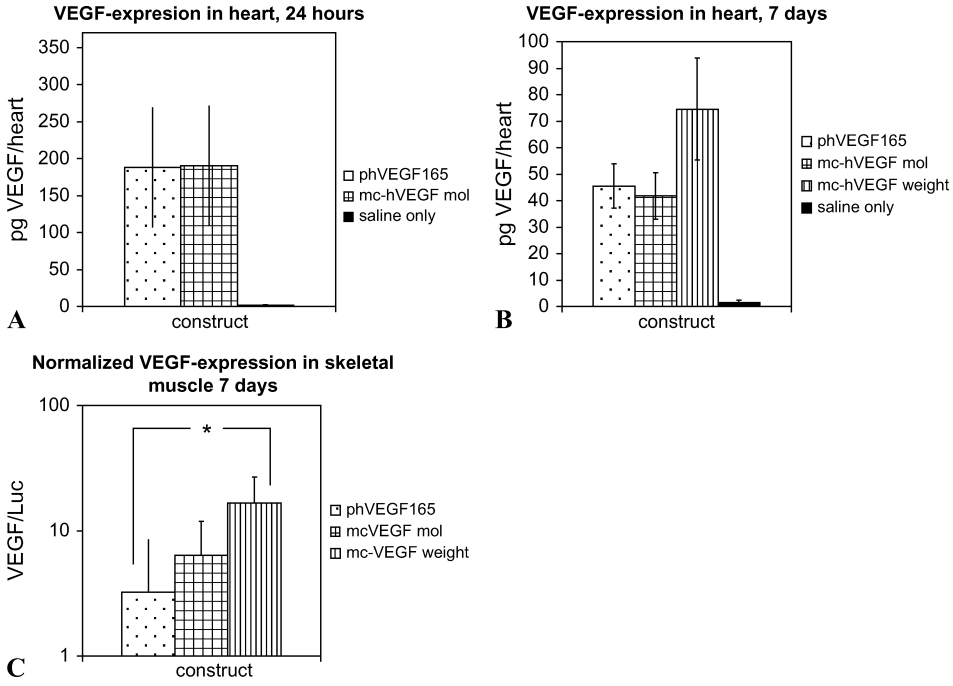

FIGURE 3. A, Gene expression of *hVEGF* from phVEGF165 (n = 6) and MC-hVEGF (n = 6), compared, mole-to-mole in mouse heart, as well as in heart injected with saline only, measured as picograms hVEGF/heart. Expression was assayed at 24 h. B, Gene expression of *hVEGF* from phVEGF165 (n = 11) and MC-hVEGF compared mole-to-mole (n = 10) and weight-to-weigh (n = 8) in mouse heart measured as picograms hVEGF/heart, as well as in heart injected with saline only. Expression was assayed at 7 days. Please note that the scale on the y axis differs in figure a and b. C, Gene expression of *hVEGF* from phVEGF165 (n = 4) and MC-hVEGF, compared mole-to-mole (n = 4) and weight-to-weight (n = 4) in mouse skeletal muscle measured as picograms *hVEGF* per millilitre normalized against Luciferase activity at day one and multiplied by 10^6. Expression was assayed at 7 days. The t-test shows that the difference between the mini-circle and the original plasmid construct in the weight-to-weight comparison is significant, ($P < 0.05$) marked with*. All values are mean + SEM. VEGF, vascular endothelial growth factor.

mouse heart muscle and in mouse skeletal muscle. This is in contrast to previous studies in mouse liver, where it has been shown that the MC construct has a prolonged gene expression compared with a conventional plasmid for up to 7 weeks.[5] This could be due to differences in promoter efficiency in different tissues. For example, Riu et al[13] have demonstrated that for the ubiquitin C promoters, the beneficial effect of using MCs in the liver is not seen. The study of expression from an MC vector in human nasopharyngeal carcinoma by Wu et al[14] shows that the MC expresses significantly up to 3 weeks after injection in vivo, albeit with a decrease after 3 days, whereas the parental plasmid used for comparison is barely detectable at day 7. They utilize lipid formulations when transfecting, whereas the mice in our study were injected with naked DNA vectors. As Riu et al[13] discuss, DNA–lipid complexes potentiate CpG-triggered immune response and the vector-bearing cell is lost. Hence, the parental plasmid containing the bacterial backbone with a higher CpG content might be more prone to activating the immune system than the smaller MC. Furthermore, Riu et al show that the silencing effect for certain promoters could be caused by the development of histone association on episomal DNA over time. No significant differences in chromatin structure between MC and plasmid could be detected after 24 hours or 7 days, but after several weeks, there was a prominent difference in the chromatin patterns.[13] Long-term *hVEGF* gene expression might not always be desirable. There have been reports of hemangioma development after implantation of myoblasts transduced with a retroviral vector overexpressing VEGF in both mouse skeletal[15] and heart muscles.[16] A study of the expression pattern from an MC construct in muscle over several weeks is therefore of interest and would supplement the long-term studies in liver and tumor tissues. However, due to the diminishing *hVEGF* expression after 7 days, we judged that the *hVEGF* is not the optimal reporter gene for a study of the expression from the MC construct in muscle over several weeks. During the work with this study, Chang et al[17] presented their results on a VEGF MC in mouse skeletal muscle. Our article corroborates their findings but in addition provides new information with regard to heart muscle and also includes a lower dose and a more long-term expression.

The conventional construct used in this study, phVEGF165, has previously been used in clinical trials[18] and has shown high *hVEGF* expression also in our hands. Nevertheless, the phVEGF165 contains sequences that can be considered unnecessary, for example, the Km resistance gene is inserted into an untranscribed ampicillin resistance gene. The MC, on the other hand, is stripped of these sequences, resulting in a less complex vector devoid of bacterial sequences. This results in a lower CpG content, the ratio of CpG content between the MC-hVEGF and the phVEGF165.SR being 3:5 and for the parental plasmid 3:8. Furthermore, the MCs are completely devoid of the immunostimulatory sequence, GTCGTT.[19,20] Lack of CpGs has recently been shown to be of importance in nonviral gene therapy for cystic fibrosis.[21] Because gene expression is similar on a molar basis, the smaller size, the absence of antibiotics resistance gene, and a lower CpG content in the final product make the MC a useful and appealing alternative vector for future cardiovascular gene therapy treatments.

CONCLUSIONS

We show that an MC construct due to its smaller size and the lack of bacterial sequences such as antibiotic resistance

genes is an interesting alternative to common plasmid vectors. The effect of the MC-hVEGF is demonstrated both in vitro and in vivo in the intended target organ, in heart muscle, and in skeletal muscle. The MC has an *hVEGF* expression pattern comparable to that of a known high-expressing plasmid, which has previously been used in clinical trials. Due to its smaller size, the same dose of DNA on weight basis equals a larger number of effective cassettes. Thus, higher molar doses of the MC compared with conventional plasmids may be given to the patient. This taken together makes the MC system a very interesting candidate tool for clinical trials targeting angiogenesis.

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