Novel Minicircle Vector for Gene Therapy in Murine Myocardial Infarction

Mei Huang, PhD; ZhiYing Chen, MD; Shijun Hu, PhD; Fangjun Jia, PhD; Zongjin Li, MD, PhD; Grant Hoyt, BS; Robert C. Robbins, MD; Mark A. Kay, MD, PhD; Joseph C. Wu, MD, PhD

Background—Conventional plasmids for gene therapy produce low-level and short-term gene expression. In this study, we develop a novel nonviral vector that robustly and persistently expresses the hypoxia-inducible factor-1 alpha (HIF-1α) therapeutic gene in the heart, leading to functional benefits after myocardial infarction.

Methods and Results—We first created minicircles (MC) carrying double-fusion reporter gene consisting of firefly luciferase and enhanced green fluorescent protein (Fluc-eGFP) for noninvasive measurement of transfection efficiency. Mouse C2C12 myoblasts and normal FVB/N mice were used for in vitro and in vivo confirmation, respectively. Bioluminescence imaging showed stable MC gene expression in the heart for >12 weeks and the activity level was 5.6±1.2-fold stronger than regular plasmid at day 4 (P<0.01). Next, we created MC carrying HIF-1α (MC-HIF-1α) therapeutic gene for treatment of myocardial infarction. Adult FVB/N mice underwent left anterior descending ligation and were injected intramyocardially with: (1) MC-HIF-1α; (2) regular plasmid carrying HIF-1α (PL-HIF-1α) as positive control; and (3) PBS as negative control (n=10/group). Echocardiographic study showed a significantly greater improvement of left ventricular ejection fraction in the MC group (51.3%±3.6%) compared to regular plasmid group (42.3%±4.1%) and saline group (30.5%±2.8%) at week 4 (P<0.05 for both). Histology demonstrated increased neangiogenesis in both treatment groups. Finally, Western blot showed MC express >50% higher HIF-1α level than regular plasmid.

Conclusion—Taken together, this is the first study to our knowledge to demonstrate that MC can significantly improve transfection efficiency, duration of transgene expression, and cardiac contractility. Given the serious drawbacks associated with most viral vectors, we believe this novel nonviral vector can be of great value for cardiac gene therapy protocols. (Circulation. 2009;120[suppl 1]:S230–S237.)

Key Words: hypoxia-inducible factor ▪ ischemic heart disease ▪ minicircle ▪ molecular imaging

One of the most important objectives in gene therapy is the development of safe and efficient systems for gene transfer in eukaryotic cells. There are 2 strategies to provide target genes for gene transfer: viral-based and nonviral-based systems. Although viral-based systems have shown high transfection efficiency in vivo, they have serious disadvantages, such as immunogenicity and inflammatory response. Nonviral gene delivery strategies are usually based on plasmid DNA carrying the gene of interest. Plasmid DNA is utilized in ~25% of all clinical gene therapy trials as of mid 2007. Conventional plasmid vectors include a bacterial backbone and a transcription unit. However, these sequences may cause undesirable effects such as the production of antibodies against bacterial proteins expressed from cryptic upstream eukaryotic expression signals, changes in eukaryotic gene expression caused by the antibiotic resistance marker, and immune responses to CpG sequences. Compared to conventional plasmids, minicircles (MC) are supercoiled DNA molecules that are smaller and that lack a bacterial origin of replication and an antibiotic resistance gene. MC are obtained in Escherichia coli (E coli) by att site-specific recombination mediated by the bacteriophage Φ3l integrase (Figure 1A). MC may contain no more than an eukaryotic expression cassette and the attR fragment resulting from the attPlattB recombination event. Thus, they constitute a safe, nontransmissible genetic material for nonviral gene therapy.5,7

Coronary artery disease is the leading cause of morbidity and mortality in the western world. One of the most efficient approaches to treat patients with coronary artery disease is to deliver potent angiogenic factors to stimulate new vessel growth. Hypoxia-inducible factor-1 alpha (HIF-1α) is known to control the expression of >60 genes that affect cell survival and metabolism in adverse conditions, including...
vascular endothelial growth factor, fibroblast growth factor, insulin-like growth factor, erythropoietin, and nitric oxide synthase, among others. With the use of various gene transfer techniques, it is now possible to modify cardiac cells to overexpress beneficial proteins or inhibit pathological proteins and achieve desired therapeutic effects. In this study, we have developed novel nonviral MC capable of transferring a reporter gene (Fluc-eGFP) and a therapeutic gene (HIF-1α).
with higher efficiency than regular plasmids both in vitro and in vivo. Our results suggest that the use of MC may offer a promising avenue for safe and efficacious nonviral-based cardiac gene therapy in the future.

Materials and Methods

Construction of MC Plasmids

For the production of MC with ubiquitin promoter-driving double-fusion (MC-DF), firefly luciferase and enhanced green fluorescent protein (Fluc-eGFP) were amplified with Fluc/eGFP (FG)-forward (5'-CCGAAATTCGAGTTCGTGCGG) and FG-reverse (5'-AAAAAAGCCGCTATCTATTCA) primers using pUbiquitin-Fluc-eGFP as a template (Figure 1B). Amplification conditions are as follow: 2 minutes at 94°C for initial denaturation, then 30 cycles of 30 seconds at 94°C for denaturation, 30 seconds at 63.4°C for annealing, and 1 minute at 68°C for extension, and then 10 minutes at 72°C for the final extension. All polymerase chain reactions were performed in MyCycler (Bio-Rad, Calif). For the production of MC with ubiquitin promoter-driving HIF-1α therapeutic gene (MC-HIF-1α), the DNA fragment that contains the ubiquitin promoter, the HIF-1α cDNA, and SV40 poly adenylation signal sequence were excised with EcoRI and XbaI from the pcDNA3.1-HIF-1α, and then bluntly ligated between the attB and attP sites of the p26c3 plasmid (Figure 1C). Note that we used a mutant version of the HIF-1α, which was created by site-specific mutagenesis to create P402 and P564 (P402A/P564G), rendering it less prone to hydroxylation and proteosomal degradation as previously described (kind gift from Dr Amato Giaccia, Department of Radiation Oncology, Stanford University Medical School).10

Preparation and Amplification of MC DNA

E. coli Top10 (Invitrogen) was transformed by parental plasmids. A single colony of the transformants was grown at 37°C overnight (OD600=4.5-5.0). The 1 L of bacterial culture in the steady state was spun down in a centrifuge (rotor JA-14, J2-MC centrifuge; Beckman, Fullerton, Calif) at 1300g for 15 minutes at 20°C. The pellet was resuspended with 1 L of fresh Lennox L Broth (pH 7.0) containing 1% L(+)-arabinose. The resuspended bacteria were incubated at 30°C with constant shaking at 225 rpm for 2 hours. Subsequently, 1 L of fresh Lennox L Broth (pH 8.0) containing 1% L(+)-arabinose was added to the culture and the bacteria were cultivated for additional 2 hours at 37°C for the activation of the restriction enzyme I-ScI1, which cuts and linearizes the bacterial backbone plasmids and subject them for degradation (Figure 1). Supercollider MC DNA was isolated from the culture using plasmid purification kits from the Qiagen (Valencia, Calif). The contaminated endotoxin in the DNA preparation was removed by the AffinityPak Detoxi-Gel (Pierce, Rockford, Ill).

Cell Culture and Transfection

Mouse C2C12 myoblast cells were cultured in DMEM containing 10% fetal bovine serum. All cells were maintained in a 5% CO2 incubator. For the transfection, cells were seeded at a density of 5×10^6 cells/well in the 6-well flat-bottom microassay plates (Falcon Co., Becton Dickenson, Franklin Lakes, NJ) 24 hours before the transfection. At 70% to 80% confluence, cells were transfected with 4 μg of plasmids carrying the double fusion reporter gene (PL-DF) or equimolar 2 μg of MC carrying the DF reporter gene (MC-DF) and incubated for an additional 48 hours. Lipofectamine 2000 (Invitrogen) was used for the transfection according to the manufacturer’s protocol.

Noninvasive Bioluminescence Imaging to Assess Duration of Reporter Gene Expression

To compare the duration of gene expression in vivo, 25 μg of PL-DF and 12.5 μg of MC-DF were injected into normal mouse hearts after aseptic open thoracotomy (n=5 per group). Bioluminescence imaging (BLI) was performed with the Xenogen In Vivo Imaging System (Alameda, Calif) on days 0, 1, 4, 7, 21, 42, 60, and 90 by an investigator blinded to study conditions (S.H.). After intraperitoneal injection of the reporter probe D-luciferin (150 mg/kg body weight), animals were imaged. The same mice were scanned repetitively according to the specific study design. BLI signals were quantified in maximum photons per second per centimeter squared per steradian (p/s/cm²/sr) as described.11 Briefly, after anesthetic induction with 2% isoflurane, reporter probe D-luciferin (Promega) was injected into the peritoneal cavity. After waiting for 10 minutes to allow D-luciferin biodistribution, the animals were placed in a light-tight chamber and baseline gray-scale body surface images were taken. Photons emitted from Fluc/D-luciferin photochemical reaction within the animal were acquired repetitively (1 to 10 minutes of acquisition time per image, 5 to 15 images per animal) until peak value was confirmed. We then averaged the 3 images with the highest p/s/cm²/sr values and used that to represent the Fluc transgene expression for that mouse on that particular day.

Animal Surgery to Induce Myocardial Infarction

Ligation of the mid left anterior descending (LAD) artery was performed in adult female FVB/N mice (Charles River Laboratories, Wilmington, Mass) by a single experienced microsurgeon (G.H.). Myocardial infarction was confirmed by myocardial blanching and EKG changes. After waiting for 10 minutes, animals were then injected intramyocardially with 12.5 μg of MC carrying HIF-1α (MC-HIF-1α), or equimolar 25 μg of regular plasmids carrying HIF-1α (PL-HIF-1α) as positive control, or phosphate-buffered saline (PBS) as negative control (n=10 per group). Injections were made near the peri-infarct region at 3 different sites with a total volume of 25 μL using a 31-gauge Hamilton syringe. Study protocols were approved by the Stanford Animal Research Committee.

Analysis of Left Ventricular Function With Echocardiogram

Echocardiography was performed before (day -7) and after (week 2, week 4, and week 8) the LAD ligation. The Siemens-Acuson Sequoia C512 system equipped with multi-frequency (8–14 MHz) 15L8 transducer was used by an investigator (Z.L.) blinded to group designation. Left ventricular end-diastolic volume and end-systole volume were calculated by the bullet method as follows: end-diastolic volume=0.85×cross-sectional area (end-diastole)×left ventricular length (end-diastole), end-systole volume=0.85×cross-sectional area (end-systole)×left ventricular length (end-systole). Cross-sectional areas are obtained from short-axis view at the level of the papillary muscles. Left ventricular length (apex to mid-mitral plane) is obtained from the parasternal long-axis view. The left ventricular ejection fraction was calculated as: left ventricular ejection fraction %=(end-diastolic volume−end-systole volume)/100×end-diastolic volume as described.12

Western Blot of Mouse Hearts to Assess HIF-1α Levels

To determine the extent of HIF-1α activation after different experimental conditions, we randomized mice to sham surgery (open thoracotomy only), ischemia-reperfusion for 30 minutes, and LAD infarction (n=3 per group). Hearts from these animals were assayed for HIF-1α levels using Western blots on week 1, week 2, and week 3. After cutting the infarction part of the left ventricle under a 10× microscope, we isolated tissue protein by Ripa buffer (Sigma). Protein concentration of lysate supernatant was determined by the DC protein assay (Bio-Rad protocol). Whole tissue extracts (25 μg) in equal volume of 2× loading buffer were run onto 10% Tris-glycine SDS-PAGE gels and transferred to Hybond ECL membrane (Amer sham). Protein blots were analyzed with rat antismouse HIF-1α (1/500 dilution; Novus) followed by sheep antirat IgG whole-antibody HRP secondary (1/3000 dilution; Amersham) and developed using ECL assay (Amer sham).
Histological Examination

Explanted hearts from study and control groups were embedded into OCT compound (Miles Scientific, Elkhart, Ind.). Frozen sections (5-μm-thick) were processed for immunostaining. To quantify the left ventricular infarct size, trichrome staining was performed in PBS, plasmid, and MC-treated hearts (n=4 per group). For each heart, 8 to 10 sections from apex to base (1.2 mm apart) were analyzed. Images were taken for each section to calculate the fibrotic and nonfibrotic areas, as well as ventricular and septal wall thickness. Scarring was determined as fibrotic area/(fibrotic+nonfibrotic area), as previously described. The NIH Image J software was used to quantify the infarct zones. To detect microvascular density in the peri-infarct area, a rat anti-CD31 (BD Pharmingen) was used. The number of capillary vessels was counted by a blinded investigator (F.J.) in 10 randomly selected areas using the picture under a fluorescent microscope (×100 magnification). A typical green vessel was selected as sample after opening 1 picture randomly by Image J software. The process was repeated 10 times in different per-infarct areas to calculate numbers at 1-mm² scale. Additional samples were used to examine the infarction size by H&E staining.

Comparison of Viral versus Nonviral Vectors

To determine the duration of gene expression and the effects of immunologic response on viral versus nonviral vectors, adult immunocompetent female FVB/N mice were injected with recombinant adenovirus-associated virus (AAV) serotype 9 carrying cytomegalovirus (CMV) promoter driving firefly luciferase (AV-CMV-Fluc at 1×10⁶ pfu; gift from Roger Hajjar, Mount Sinai School of Medicine), 12.5 μg of MC-DF, or 25 μg of PL-Fluc in the right leg. Animals were imaged on days 1, 5, 7, 14, 21, and 28 using the Xenogen IVIS system. Afterward, the same animals were injected with equal dosage of the same vectors into the left legs and imaging was performed at the same time points.

Statistical Analysis

ANOVA and repeated-measures ANOVA with post hoc testing as described. Differences were considered significant at P<0.05.

Statement of Responsibility

The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Intramolecular Recombination Splits Parental Plasmid Into MC and Bacterial Backbone

MC are the product of site-specific intramolecular recombination between the attB and attP sites driven by bacteriophage φC31 integrase. After isolation from the bacterial backbone (Figure 1A), the MC now lack both an origin of replication (cannot self-replicate) and an antibiotic selection marker (cannot confer resistance to other microorganisms), and carry only short bacterial sequences (greatly limiting immune responses against CpG sequences in the bacterial backbone). In this study, we first constructed MC-DF that allowed us to determine the transfection efficiency (in C2C12 cells) and duration of transgene expression (in living animals). The Fluc-eGFP cDNA was successfully cloned by polymerase chain reaction and ligated into the parental plasmid p2φc31.UB-DF (Figure 1B). We next constructed MC-HIF-1α that allowed us to assess the therapeutic efficacy of MC versus PL-based gene therapy approaches (Figure 1C). The HIF-1α gene was successfully ligated into the parental plasmid p2φc31.UB-HIF-1α. We used the ubiquitin promoter (UB) for both parental plasmids because it has been shown to drive high levels of transgene expression with minimal gene silencing. By addition of arabinose at 32°C, the phage φC31 integrase performs a site-specific intramolecular recombination of sequences between attB and attP recognition sites, splitting p2φC31 parental plasmid into 2 supercoiled circular DNAs: the MC with its transgene of interest and the remaining DNA with a “junk” bacterial backbone. To physically separate the 2 end products, we then adjust the pH and temperature to pH 8.0 and 37°C, respectively. The bacterial backbone plasmid is linearized by the induced I-Scel and degraded by bacterial exonucleases (Figure 1B,C). As a consequence, only the MC containing the transgene of interest in our study (DF and HIF-1α) remained intact as an episome formation in the bacterial cytosol, which can then be isolated for subsequent usage.

Evaluation of Novel MC versus Regular Plasmids in Cell Line

To assess the transfection efficiency, equimolar amounts of MC-DF and PL-DF were used to transfect mouse C2C12 cells. Fluc was evaluated by BLI (Figure 2A) and eGFP was evaluated by flow cytometry (Figure 2B). MC-DF showed 5.5±1.7-fold (at 12 hours) and 8.1±2.8-fold (at 48 hours) higher Fluc expression compared to PL-DF. Because the DF is a fusion construct whereby the 2 reporter genes are linked by a 5-amino acid linker (GSHGD), MC-DF also showed earlier onset of eGFP activity by 12 hours (8.16±0.04 versus 0.23±0.03%; P<0.01), as expected. Overall, these data demonstrate MC can mediate faster and higher transgene expression in vitro than regular plasmids.

Comparison of Novel MC versus Regular Plasmids in Living Animals

To determine expression level in vivo, we next injected MC-DF and PL-DF into normal mouse hearts and followed their gene expression from day 0 to day 90 using BLI (Figure 3A). Quantitative analyses of Fluc activities for both groups are shown in Figure 3B. Overall, mice injected with MC-DF had significantly higher Fluc activity compared to mice injected with PL-DF at day 1 (2.6×10⁴±7.9×10³ versus 1.4×10⁴±1.3×10³; P<0.0001), day 7 (3.2×10⁴±1.5×10⁴ versus 6.8×10³±4.2×10³; P<0.0001), day 14 (2.8×10⁴±2.6×10³ versus 9.7×10³±8.6×10³; P<0.0001), day 28 (8.9×10³±4.1×10³ versus 1.6×10³±3.9×10³; P<0.0001), day 42 (7.4×10³±8.7×10³ versus 1.3×10³±2.9×10³; P<0.0001), day 60 (6.5×10³±5.7×10³ versus 1.3×10³±3.8×10³; P<0.0001), and day 90 (4.4×10³±2.1×10³ versus 1.5×10³±3.1×10³ p/s/cm²/sr; P<0.0001). Overall, these data demonstrate MC can mediate stronger and longer transgene expression in vivo than regular plasmids.

Injection of MC Carrying HIF-1α Improved Cardiac Function After Myocardial Infarction

To examine whether using MC-HIF-1α can also improve cardiac function after myocardial infarction, echocardiography was performed before (day −7) and after (week 2, week 4, and week 8) the LAD ligation. At day −7, the left ventricular ejection fraction was comparable in all 3 groups (Figure 4). After LAD ligation, the MC group had significantly higher ejection fraction compared to the PBS control group at both week 4 and week 8 (P<0.01 for both). The regular
plasmid group had significantly higher ejection fraction compared to the PBS group at week 4 (plasmid: 42.2±6.7% versus PBS: 35.1±5.5%; P=0.004). However, this beneficial effect was no longer present by week 8 (42.8±6.0% versus 37.8±3.4%; P=0.38). This is likely attributable to the short-term transgene expression of regular plasmids (4 weeks) as shown by our imaging results (Figure 3A).

Ex Vivo Histological Validation of In Vivo Imaging Data
After imaging, all animals were euthanized and hearts were explanted. H&E staining showed thicker heart wall size for the MC group compared to regular plasmid group and saline group at week 4, confirming the positive functional imaging data seen in echocardiography (Figure 5A). MC treatment significantly decreased left ventricular scarring compared to plasmids and PBS control (12.85±1.32% versus 21.5±3.51% versus 31.2±3.58%; P=0.045 MC versus PL; P=0.001 MC versus PBS). Immunohistochemistry of the peri-infarct region by CD31 staining also showed increased neovascularization in the MC (389±73.1) compared to the regular plasmid (251±21.5) and PBS (138±9.6 vessels/mm²) groups (P<0.05 for both; Figure 5B). To further confirm the

Figure 2. Comparison of MC versus regular plasmids in vitro. A, The DF consists of Fluc and eGFP linked by a 5-amino acid linker (GSHGD). In vitro BLI shows that Fluc signals are significantly higher in C2C12 cells transfected with MC compared to plasmids at all time points. B, Quantitation of Fluc indicates that MC are 5.5±1.7 (at 12 hours) and 8.1±2.8-fold (at 48 hours) higher than regular plasmid. Note the difference in y-axis bars between the 2 plots. C, The eGFP expression through FACS at 12 hours coincides with the bioluminescence imaging results.

Figure 3. Comparison of MC versus regular plasmids in vivo. A, Both MC-DF and PL-DF were injected into normal murine hearts. Mice injected with MC (top row) showed more robust Fluc signals compared to mice injected with regular plasmid (bottom row). Transgene expression was detectable at day 1, peaked at week 1 to 2, and lasted for >90 days. B, Detailed quantitative analysis of Fluc bioluminescence signals from days 1 to 28 (left) and days 28 to 90 (right). Note the difference in y-axis scale bars (as p/s/cm²/sr) between the 2 plots. Background bioluminescence signal is denoted by the dashed line (1.33×10⁵ p/s/cm²/sr).
in vivo functional imaging data, we assayed for HIF-1α protein expression of explanted hearts at day 14. Quantitative analysis of the Western blot indicates that HIF-1α proteins were significantly higher in the MC-treated hearts compared to regular plasmids and PBS-treated hearts (Figure 5C,D). Finally, we also investigated HIF-1α expression levels in different ischemia conditions as well as in different time points after LAD ligation. Western blot data show that endogenous HIF-1α is most robust after LAD ligation compared to ischemia-reperfusion and sham surgery, suggesting that activation of endogenous HIF-1α expression level is directly related to the size of myocardial infarction (Figure 5E). With coadministration of MC-HIF-1α, highest levels of HIF-1α are detected at week 1. The levels of MC-treated HIF-1α decreases at subsequent weeks 2 and 3, similar to the in vivo imaging pattern that was observed for Fluc transgene expression in Figure 3A.

**Comparison of Viral versus Nonviral Vectors in FVB/N Mice**

Adenoviral vectors carrying either vascular endothelial growth factor or fibroblast growth factor have been used for several cardiac gene therapy trials. However, our group has previously shown that repeated injection of adenovirus in-
ducens a significant host cellular and humoral immune response. Recently, AAV has generated significant interest as a safer and more effective vehicle for cardiac gene transfer. AAV carrying sarcoplasmic reticulum calcium ATPase (SERCA2a) has been used for treatment of patients with heart failure. Here we investigated the duration of gene expression after repeated intramuscular transplantation of AAV versus MC and regular plasmid into immunocompetent adult female FVB/N mice (n = 5 per group). Figure 6 shows that AAV-mediated Fluc expression was significantly higher compared to MC and PL after first injection as expected. At day 7, the activities were 1.42 × 10⁴ ± 8.65 × 10³ versus 7.46 × 10³ ± 5.52 × 10³ versus 2.85 × 10⁵ ± 9.46 × 10⁴ p/sec/cm²/sr for each group, respectively (P < 0.05 AAV versus MC and P < 0.001 AAV versus PL). At day 28, the activities were 2.31 × 10⁴ ± 6.3 × 10³ versus 3.34 × 10⁵ ± 7.25 × 10⁴ versus 1.88 × 10⁵ ± 3.51 × 10⁴ p/sec/cm²/sr, respectively (P < 0.05 AAV versus MC and P < 0.001 AAV versus PL). However, repeat administration of AAV in the contralateral leg at 4 weeks after primary injection resulted in no AAV-mediated signal expression. At day 28, the BLI signals for MC were comparable between first and second injection (3.34 × 10⁴ ± 7.25 × 10³ versus 2.15 × 10⁴ ± 3.83 × 10³; P = NS), whereas the BLI signals for AAV were significantly reduced (2.31 × 10⁴ ± 6.31 × 10³ versus 1.12 × 10⁵ ± 6.46 × 10⁴ p/sec/cm²/sr; P < 0.0001). Taken together, these data suggest that AAV is capable of triggering both cellular and humoral immune response, which are consistent with 2 previous reports of dose-dependent generation of CD8⁺ T-cell responses to AAV capsid proteins in clinical trials involving patients with hemophilia B due to factor IX deficiency and patients with inherited hypertriglyceridemia due to lipoprotein lipase deficiency.

**Discussion**

Although most phase 1 clinical trials in patients with myocardial ischemia provided encouraging results, more recent phase 2 randomized trials (AGENT, VIVA, and KAT) yielded inconsistent or modest benefits at best. These inconsistencies have been attributed to the lack of ideal vectors, inability to monitor gene transfer in vivo, and the unclear role of single therapeutic genes such as vascular endothelial growth factor for inducing neoangiogenesis. In this article, we addressed these 3 issues by using a novel nonviral vector carrying a more robust therapeutic gene and validating the results with molecular imaging technology. The major findings are as follows. First, MC can be easily isolated from the parental plasmid in E. coli culturing medium. In our study, we used the I-sceI site, a Φc31 recombinase temperature-sensitive recognition and cutting site to obtain large amounts of purified MC. Second, MC show earlier onset and more robust transgene expression than conventional plasmids both in vitro and in vivo. In particular, flow cytometry data showed significant eGFP positivity by 12 hours compared to normal plasmids. BLI shows that MC-DF injected into murine hearts lasted >90 days, much longer than the 28 days seen in regular plasmids. Third, direct injections of MC-HIF-1α can improve ventricular function and enhance neoangiogenesis in a mouse model of myocardial infarction for 8 weeks, compared to 4 weeks for regular plasmids. Last, repeated injections of MC have comparable transgene activities. In contrast, repeated injections of AAV lead to significant reduction of transgene expression attributable to host cellular and humoral immune response.

Nonviral vectors have many advantages over viral systems, such as a better safety profile, the absence of theoretical size limitation for the expression cassette, and possibly simpler clinical translation attributable to easier good manufacturing practices. However, concerns have been raised regarding the lack of robust transfection efficiency and the immunostimulatory prokaryotic CpG motives in the bacterial backbone. To resolve these issues, we designed the novel nonviral MC plasmids that exhibited up to 8-fold higher gene expression than the regular plasmid in vitro, as well significantly longer transgene expression in vivo. This drastic improvement is attributable to the removal of unnecessary plasmid sequences, which could affect the gene expression, and the smaller size

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![Figure 6. Comparison of viral versus nonviral mediated gene expression.](image-url)
of the MC, which might confer better extracellular and intracellular bioavailability and thus improved gene delivery properties.\(^{21,22}\) In particular, the bacterial backbone sequences are often abundant with CpG islands, which can lead to transcriptional gene silence in vivo, and is one of the major reasons why regular plasmids are notoriously ineffective for long-term expression.\(^{23}\)

The HIF-1 \(\alpha\) complex is known to control the expression of >60 genes that affect cell survival and metabolism in adverse conditions.\(^{9}\) As an upstream transcriptional factor, HIF-1 \(\alpha\) is involved in activation of several pathways.\(^{24}\) Overexpression of HIF-1 \(\alpha\) has critical functional consequences, including an improvement in neoangiogenesis attributable to upregulation of vascular endothelial growth factor, fibroblast growth factor, and endothelial nitric oxide synthase.\(^{25,26}\) Importantly, recent evidence suggests that the expression of a single angiogenic factor such as vascular endothelial growth factor alone may not be sufficient for the functional revascularization of ischemic tissues.\(^{27}\) Thus, newer approaches based on upregulation of the master regulator HIF-1 \(\alpha\) may be a more potent choice. In this study, we selected HIF-1 \(\alpha\) as a therapeutic gene, which resulted in significant functional improvements after delivery into the infarcted hearts. Immunohistochemical results demonstrated that higher HIF-1 \(\alpha\) expression led to formation of more small vessels, which in turn improved cardiac function.

In summary, MC are novel nonviral vectors that lack an origin of replication and an antibiotic selection marker, and carry only short bacterial sequences. Our results suggest that using MC to deliver HIF-1 \(\alpha\) may represent a potentially new therapeutic target in the field of cardiovascular gene therapy.

**Sources of Funding**

This work was supported in part by grants from the NIH HL093172, NIH DP2 OD004437, AHA 0970394N, Baxter Faculty Scholar Award (J.C.W.) and AHA Postdoctoral Fellowship (M.H.).

**Disclosures**

None.

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Circulation. 2009;120:S230-S237
doi: 10.1161/CIRCULATIONAHA.108.841155
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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