Vascular endothelial growth factor-A gene electrotransfer promotes angiogenesis in a porcine model of cardiac ischemia

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This study aimed to assess safety and therapeutic potential of gene electrotransfer (GET) as a method for delivery of plasmid encoding vascular endothelial growth factor A (VEGF-A) to ischemic myocardium in a porcine model. Myocardial ischemia was induced by surgically occluding the left anterior descending coronary artery in swine. GET following plasmid encoding VEGF-A injection was performed at four sites in the ischemic region. Control groups either received injections of the plasmid without electrotransfer or injections of the saline vehicle. Animals were monitored for 7 weeks and the hearts were evaluated for angiogenesis, myocardial infarct size and left ventricular contractility. Arteriograms suggest growth of new arteries as early as 2 weeks after treatment in electrotransfer animals. There is a significant reduction of infarct area and left ventricular contractility is improved in GET-treated group compared with controls. There was no significant difference in mortality of animals treated with GET of plasmid encoding VEGF-A from the control groups. Gene delivery of plasmid encoding VEGF-A to ischemic myocardium in a porcine model can be accomplished safely with potential for myocardial repair and regeneration.

INTRODUCTION

Heart disease is the leading cause of death in the United States accounting for a quarter of all deaths.1–4 Approximately 22% of men and 46% of women surviving myocardial infarction (MI) will develop congestive heart failure within 6 years after MI.1–3 Of those diagnosed with heart failure, the 5-year survival rate is only 50%.1–4 Extensive atherosclerosis of the coronary arteries can lead to sufficient blockage of the coronary vessels to cause downstream ischemia in the ventricles of the heart.5 Therefore, current clinical therapies aim at direct revascularization to improve the survival rates for patients after MI.5 However, the myocardium does not regenerate to its former electrical, histological and functional pumping potential.1–5 Rather, ischemic myocardium remodels with fibroblast proliferation and collagen deposition replaces cardiomyocytes, resulting in thin, non-contracting patches in the ventricle, limiting cardiac output (CO).6 There is little clinical evidence of return of viable cardiomyocytes in patients surviving MI.5–8

Because of atherosclerosis, angiogenesis in ischemic myocardium is likely impaired. There are many preclinical therapies aimed at inducing angiogenesis in ischemic myocardium. Vascular endothelial growth factor A (VEGF-A) is a commonly used gene for therapeutic angiogenesis in coronary artery disease models, with intentions of collateral vessel development in hypoxic areas.9 The primary role of VEGF-A is induction of angiogenesis by stimulation of endothelial cells to migrate and proliferate in hypoxic areas.9–11 VEGF-A also has an important role in stem cell mobilization and differentiation to cardiomyocytes as well as cardiomyocyte proliferation.12–15 VEGF-A promotes embryonic stem cell differentiation into cardiomyocytes in a mouse model16 and has been shown to promote cardiac stem/progenitor cell mobilization and cardiomyocyte differentiation12 and cardiomyocyte proliferation.13 In addition to the use of VEGF-A delivered in the form of recombinant protein, several gene therapy approaches for VEGF-A delivery to the myocardium have also been evaluated.6

Primary methods of gene delivery are viral-mediated transfer and plasmid-based transfer. Adenoviral vectors and aden-associated vectors are the most commonly used vectors for delivery to the heart with immunogenicity and toxicity as major side effects. Plasmid DNA delivery is associated with fewer side effects, low immune response, low toxicity and reduced cost but with lower efficiency.6,16,17 There are several studies reported on electroporation aka gene electrotransfer (GET)-mediated gene delivery to the beating heart in a small animal model.18,19 In our previous studies, we reported on plasmid DNA delivery via GET to cardiac muscle in vivo in a porcine model.20,21 GET of plasmid DNA can be applied safely directly to the ischemic and non-ischemic myocardium and gene expression can be modulated by pulsing conditions.20,21 We have also determined that there may be a therapeutic benefit to local delivery of pVEGF-A to ischemic myocardium, with improved myocardial perfusion 2 weeks after treatment.21

Our current study is focused on long-term outcome of GET delivery of pVEGF-A to ischemic myocardium. We demonstrate that 7 weeks after treatment new arteries can be observed in arteriograms in the majority of treated ischemic porcine hearts. We also observed a reduction of myocardial damage from ischemic injury at treatment sites, with improved contractility and relaxation of the left ventricular myocardium under chemically induced stress. Overall, our results indicate a potential long-term therapeutic benefit of exogenous VEGF-A gene delivery mediated by electrotransfer to ischemic myocardium, with
RESULTS

Occlusion of left anterior descending (LAD) coronary artery leads to MI of anterior left ventricle

To study the effects of an angiogenic therapy for MI, we first confirmed the presence of MI of the anterior left ventricle after occlusion of the LAD coronary artery. Thrombosis in myocardial infarction (TIMI) scoring of the arterial tree confirmed successful permanent occlusion of the LAD coronary artery (Figure 1a). There were two animals that were excluded from the study based on a TIMI score of 3, indicating insufficient occlusion, and lack of ischemia (which was confirmed at 7 weeks, with lack of MI detectable by triphenyltetrazolium staining). SPY System analysis indicates the presence of a consistent ischemic region in terms of size and drop in perfusion (Figures 1b and c). Further analysis of plasma indicated a sharp increase of cardiac troponin-I 24 h after the induction of ischemia, indicating the presence of MI (Supplementary Table S1). There is a sharp increase of plasma creatine kinase muscle-isoform (CK-MM), indicating muscle cell damage and also consistent with MI in all the groups (Supplementary Table S1).

GET of pVEGF-A to the ischemic myocardium has no deleterious effects on survival or CO in a porcine model

Kaplan–Meier log-rank survival analysis was performed on all animals surviving the onset of acute ischemia and receiving GET with pVEGF-A, pVEGF-A injections without electrotransfer or saline injections. Two animals were excluded from the study owing to irreversible ventricular fibrillation following the occlusion of the LAD coronary artery prior to any experimental treatment. Two additional animals were excluded owing to lack of sufficient occlusion (TIMI score of 3), resulting in unabated flow through the coronary artery, thus insufficient ischemia and no MI. There was no significant difference in survival between treated groups and untreated controls (Figure 2a), indicating no significant increase of mortality owing to electrotransfer-mediated gene delivery of VEGF-A encoding plasmid DNA to ischemic myocardium 7 weeks after induction of ischemia.

Measurements of left ventricular diameters via echocardiography indicate no significant difference in calculated end-systolic and end-diastolic measures of volumes, ejection fraction, fractional shortening and CO between GET treated, pVEGF-A injection without electrotransfer and sham control groups at any time point throughout the study (Figures 2b–f), indicating no additional damage caused by GET-mediated pVEGF-A to ischemic myocardium of the left ventricle. Other cardiac ultrasound parameters are also consistent with this observation (Supplementary Figure S1).

GET of pVEGF-A induces angiogenesis in ischemic myocardium in a porcine model

Arteriograms taken prior to LAD coronary artery occlusion were compared with arteriograms taken 2 and 7 weeks after LAD coronary artery occlusion and gene therapy treatment. More than 70% of GET treated animals developed new arteries large enough to be visible via arteriogram of the heart, whereas only 25% and 40% of the pVEGF-A injection only and saline injection controls, respectively, developed new vessels (Figures 3a and b). The average length of new vessels as visible in arteriograms was not significantly different between the treatment groups for animals that did have new vessel growth (Supplementary Figure S2).

Histological comparison of vessel density in 10 randomly selected fields of view from treatment sites indicate no significant difference in blood vessel density between the groups 7 weeks after induction of MI and gene therapy treatment (Figures 3c and d).

GET of pVEGF-A reduces myocardial damage from ischemic injury at treatment sites of the left ventricle

MI caused by occlusion of the LAD coronary artery extended through the anterior left ventricle, septum and right ventricle in most animals, based on triphenyltetrazolium staining performed on harvested hearts 7 weeks after LAD coronary artery occlusion and gene therapy treatment (Supplementary Figure S3). The infarct/ischemic area in the left ventricle after LAD occlusion is significantly lower in GET of the pVEGF-A group than the saline injection group and is lower than the pVEGF-A injection without electrotransfer (Figure 4b). Therefore, the infarct size in GET with the pVEGF-A group at the treatment sites is smaller than the infarct size of the control groups. The total volume of MI was not significantly different between the experimental groups (Figure 4a). There is notable variability in the extent of the infarction into the right ventricle and septum, which were untreated affecting the total size of the infarct (Supplementary Figure S3).

GET of pVEGF-A improves left ventricular contractility and relaxation under dobutamine stress

Although the heart rate of all animals irrespective of the treatment group increased dramatically in response to dobutamine administration, the change in intraventricular pressure correlated inversely with total size of infarct in the left ventricle. Higher ventricular compliance was observed for GET (n = 2) treated hearts than controls (n = 3), based on higher ±dP/dtmax measurements as

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Figure 1. Permanent ischemia was induced via LAD occlusion. (a) TIMI flow index drops significantly after occlusion, with no significant difference in TIMI between the treatment groups (two-way analysis of variance (ANOVA) and Tukey’s multiple comparisons test, α = 0.05, P < 0.0001). (b) SPY-based measurement of ischemic surface area of the left ventricle indicates consistent occlusion between the treatment groups, with no significant difference in ischemic area (one-way ANOVA, α = 0.05, P = 0.6388). (c) SPY-based measurement of drop in perfusion in the ischemic area of the left ventricle indicates a consistent reduction in perfusion, with GET group receiving a slightly larger drop in perfusion than the IO group (one-way ANOVA, Tukey’s multiple comparisons test, α = 0.05, P = 0.0012).

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indicators of contractility and relaxation in response to increasing dobutamine dose (Supplementary Figure S4).

**DISCUSSION**

Atherosclerosis of coronary arteries with stable or unstable atherosclerotic plaques often leads to ischemia of the myocardium or acute MI, respectively. Sufficient hypoxic conditions result in loss of cardiomyocytes and remodeling of the myocardium into non-contractile scar tissue. Sufficient loss of contractile function of the heart leads to heart failure and reduced long-term survival. Restoration of blood flow is a common strategy for clinical treatments such as coronary artery bypass grafting; however, such procedures do not address the problem of atherosclerosis. Autologous blood vessels (for example, saphenous vein), often used for bypassing blocked arteries that suffer the same atherosclerotic disease as the original vessels, can and often do develop atherosclerotic plaques. An angiogenic therapy is an attractive option owing to the potential to alleviate ischemia in short term and to cultivate new autologous vessels, which are not

Figure 2. GET of VEGF-A has no deleterious effects on survival or heart performance over 7 weeks after MI and gene delivery. (a) Kaplan–Meier survival curve with no significant difference between the groups (log-rank test for trend, \( P = 0.4639 \)), (b) ejection fraction \( (P = 0.9240) \), (c) CO \( (P = 0.5603) \), (d) fractional shortening \( (P = 0.3775) \), (e) left ventricular end systolic volume \( (P = 0.5603) \) and (f) left ventricular end diastolic volume \( (P = 0.92) \), with no significant difference between the treatment groups based on echocardiographic analysis (two-way analysis of variance and Tukey’s multiple comparison test, \( \alpha = 0.05 \)).
yet effected by atherosclerosis, similar to blood vessels of younger individuals, who are not likely to suffer from atherosclerosis. Here we demonstrate an angiogenic gene therapy approach that results in the development of new arteries in ischemic myocardium with minimal adverse effects.

Our gene-delivery approach is non-viral and non-integrating with temporary elevation of local gene expression up to 2 weeks. We injected naked plasmid DNA encoding human VEGF-A to four sites in the ischemic myocardium in a porcine animal model, followed by electrotransfer. In our previous studies, we have shown that GET greatly enhances gene expression of VEGF-A compared with plasmid DNA injection without electrotransfer in a porcine model of MI. Here we evaluated the long-term impact of GET-mediated delivery of pVEGF-A on ischemic myocardium.

The occlusion of the LAD coronary artery resulted in consistent ischemia of the left ventricle in terms of the area affected and drop in perfusion observed, both measured with SPY perfusion analysis system. There were no significant differences between ischemia of the left ventricle between the groups of animals. Ischemia of the septum and the right ventricle were not assessed, owing to limitations of the SPY imaging system. Only the apical surface of the left ventricle is visible via the SPY system, thus only perfusion through that area can be assessed before and after the occlusion. Elevated plasma levels of cardiac troponin-I and CK-MM 1 day after LAD coronary artery occlusion indicated that induction of ischemia also resulted in MI. Therefore, the occlusion of the LAD coronary artery resulted in consistent ischemia and acute MI for all the treatment groups.

Seven-week survival was evaluated with the Kaplan–Meier log-rank evaluation. Mortality was not significantly different between the treatment groups, indicating that GET of VEGF-A did not adversely affect survival or increase mortality compared with sham in the DNA injection only groups. This finding is consistent with our previous studies, indicating that proper administration of electric pulses for gene therapy during the rise of the R-wave, does not increase mortality, neither does over-expression of pVEGF-A at treatment sites of ischemic myocardium. Therefore, the GET of pVEGF-A can be safely administered to ischemic myocardium as a potential therapeutic.

Echocardiography evaluation of ejection fraction, fractional shortening, end-systolic and end diastolic volumes, CO and hypertrophy indicates no significant difference between the treatment groups at any time points of the study. The occlusion distal to the second diagonal branch resulted in a relatively mild form of ischemia leading to low mortality and allowing for evaluation of treatment effects. Variability in ischemia to the septum and the right ventricle may have a role in lack of

Figure 3. GET of pVEGF-A induces angiogenesis in ischemic myocardium at treatment sites. (a) Arteriograms of GET and IO animals, before occlusion, immediately after occlusion and 7 weeks after occlusion, with new arteries visible in the GET heart. *LAD occlusion site, red arrows point to new vessels. (b) The proportion of hearts with new arteries visible via arteriogram 2 ($P = 0.152313$) and 7 weeks ($P = 0.243917$) after treatment is higher in the hearts treated with GET of pVEGF-A (chi-square test). (c) von Willebrand factor staining for blood vessels at a treatment site of GET of pVEGF-A 7 weeks after MI and treatment. (d) Blood vessel density at treatment sites 7 weeks after MI and treatment is not significantly different between the treatment groups on a microscopic level (one-way analysis of variance, $\alpha = 0.05$, $P = 0.9704$).
Significant differences in end-systolic and end-diastolic diameters and calculated volumes, ejection fraction and CO measures between the groups. Increase in CO, end-systolic and end-diastolic volume at 7 weeks compared with the pre-MI values can also be caused by growth of the entire animal and the heart, as most animals grew throughout the duration of the study. Significantly, however, there were no adverse effects observed via echocardiography in GET of VEGF-A-treated animals compared with the control animals, indicating safety of the treatment.

Although damage to the myocardium from ischemia occurs within hours of the onset of ischemia, with some indication that necrosis of cardiomyocytes can also start within hours after the onset of ischemia, angiogenesis is not induced in the remodeling myocardium until about 2–3 weeks from MI onset, with maturation of the new vessels taking even longer. An angiogenic therapy with an onset earlier than what happens during the endogenous course of remodeling of ischemic myocardium may prove to be extremely beneficial therapeutically. Here we note the presence of new arteries in the distal regions of the ischemic myocardium as early as 2 weeks after occlusion of the LAD coronary artery (Figure 3). Arteriogram analysis (Figures 3a and b) indicates the presence of arteries distal to the location of the occlusion in the left ventricle. These vessels were not present in the arterial tree of the same hearts prior to occlusion and treatment, indicating angiogenesis in >70% of the hearts treated with GET of pVEGF-A at 7 weeks. In comparison, only ~25% and ~40% of the hearts treated with injection of pVEGF-A or saline, respectively, developed new arteries in the same time frame (Figure 3b). Arteriograms can only detect macroscopically sized vessels with radiopaque dye, thus onset of angiogenesis is actually earlier than 2 weeks, in order to observe functional arteries via arteriogram analysis (Figures 3a and b). Also, owing to the size (approximately 0.5–1 mm inner diameter) and abundant perfusion of the detected arteries, it is likely that these new collaterals arteries are mature and efficient.

Histological analysis of blood vessel density was only performed at 7 weeks for observation of long-term effects of GET of pVEGF-A on the myocardium (Figure 3c). By 7 weeks, vessel density at the treated sites is indistinguishable between the gene therapy group and corresponding controls (Figures 3c and d). Based on our previous data, exogenous gene expression is expected to persist for up to 2 weeks, therefore homeostatic processes are expected to take over after exogenous gene expression subsides. Blood vessel density generally depends on oxygen requirements of the tissue; therefore once hypoxic conditions and/or exogenous growth factors no longer drive angiogenesis, it is likely that the tissue no longer requires angiogenic processes. This is consistent with our results of similar blood vessel density on the microscopic level at 7 weeks after LAD coronary artery occlusion and gene therapy application.

VEGF-A has a well-known role in angiogenesis in response to hypoxic conditions; however, there are also multiple reports of VEGF-A promotion of cardiomyocyte proliferation as well as induction of differentiation of cardiac progenitor cells or cardiac stem cells into cardiomyocytes. Evaluation of the total size of the infarct including the myocardium of the right ventricle, septum and left ventricle yielded no significant differences between the treatment groups; however, there was considerable variability in the degree to which the right ventricle and the septum were effected by LAD coronary artery occlusion (Supplementary Figure S3), with some hearts having larger or smaller infarcts in those regions. Neither the right ventricle nor septum was evaluated for ischemia prior to treatment with SPY analysis. These regions contained no treatment sites, thus the variability in myocardial infarct size in these regions is not due to the treatment group (Figure 4a). This variability may also account for little distinction between the treatment groups via 7-week echocardiography evaluation (Figures 2b–f and Supplementary Figure S1). Therefore, we evaluated ischemia and infarct size of the left ventricle separately from other infarcted regions in order to assess the effect of GET of VEGF-A at the treatment sites. Treatment sites were only present in the left ventricle. There was a significant decrease in infarct size in terms of surface area of infarct at 7 weeks relative to the initial area of ischemia of the left ventricle, which is the site of GET treatment (Figure 4b). The reduction of myocardial infarct area of the left ventricle was larger in the GET of VEGF-A group than the pVEGF-A injection only or the saline injection (sham) control groups. The difference between GET and sham group is statistically significant (P = 0.0349), implicating a therapeutic potential for GET of pVEGF-A treatment.

A strong correlation between the presence of new arteries and reduction of infarct area was not observed, suggesting another role for exogenous VEGF-A in repair of the myocardium after ischemic injury. VEGF-A may preserve viability of resident cardiomyocytes, induce cardiomyocyte proliferation in adjacent normoxic regions, promote migration and differentiation of cardiac stem/progenitor cells to cardiomyocytes or a combination of these three. It has been noted that VEGF-A is capable of promoting proliferation of cardiomyocytes and induction of differentiation of cardiac stem cells or progenitor cells to...
cardiomyocytes. This role of VEGF-A may account for the reduction of myocardial infarct size in vivo with recruitment of new cardiomyocytes to the hypoxic myocardium in addition to the traditional angiogenic role.

Ventricular contractility and relaxation are extremely important to quality of life. The ability of the heart to respond to increased demand in oxygen owing to stress such as exercise determines patients’ daily physical abilities. Therefore, we evaluated contractility and relaxation of infarcted hearts under increasing dobutamine stress 7 weeks after treatment. GET-treated animals had a higher contractility and relaxation in response to increased stress than the controls (Supplementary Figures S4A and B). Although our sample size is very limited, these results are consistent with findings that there are more contractile cells present in the treated left ventricles, leading to improved contractility of the left ventricle in the treated groups, compared with the control groups.

In summary, GET of VEGF-A encoding plasmid applied to left ventricular ischemic myocardium has no deleterious effects on ventricular contractility and relaxation of infarcted hearts under increasing dobutamine stress 7 weeks after treatment. GET-treated animals had a significantly larger, indicating a potential therapeutic benefit for this gene therapy approach. Improved left ventricular contractility and relaxation under dobutamine stress further support the therapeutic application potential of GET of pVEGF-A to ischemic myocardium for increasing angiogenesis and myocardial repair and regeneration. This study supports safety and therapeutic potential for using GET to deliver plasmid DNA encoding VEGF-A to ischemic myocardium in a porcine model.

MATERIALS AND METHODS

Animals

Thirty-seven adult Yorkshire pigs were purchased from Bellview Farms, Smithville, VA, USA at an approximate weight of 31-47 kg. All experimental studies followed an approved Old Dominion University’s Institutional Animal Care and Use Committee protocol, in accordance with the Guide for the Care and Use of Laboratory Animals at an AAALAC-accredited facility, including a 12:12-h light cycle. An ACLAM board-certified veterinarian determined that all animals were free of disease through the use of health examinations. Animals were quarantined and acclimated for a 7-day period before any procedures were conducted. In addition, an echocardiography exam was conducted on each animal to obtain baseline measures of cardiac health.

Plasmid

A commercially prepared plasmid, pVax1-hVEGF(A), was used for these studies (Aldevron, Fargo, ND, USA). DNA was suspended in sterile saline at 2 mg ml−1. Endotoxin levels were < 0.1 EU per μg plasmid, confirmed by Aldevron via a Limulus Amebocyte Lysate assay.

Myocardial infarction

Surgical procedure and induction of acute ischemia were performed as previously described,21 with more details available in Supplementary Methods. After induction of anesthesia by ketamine (20 mg kg−1) and diazepam (3–5 mg kg−1), pigs were anesthetized with isoflurane via a nose mask and intubated, with respiration maintained by a volume-controlled DRE Bonair anesthesia ventilator (DRE Medical Inc., Louisville, KY, USA). A median sternotomy incision was made for exposing the heart. The sternotomy was chosen over less invasive methods, to allow adequately large field of view of the entire ischemic surface area for SPY measurements. The pericardium was removed to expose LAD coronary artery. The LAD was ligated with a 4-0 silk suture below the second diagonal branch, inducing downstream ischemia, with the suture left in place following treatment. A radiopaque thread was tied to the silk ligature for marking the LAD occlusion site in X-ray imaging. Ischemic areas of the heart wall were identified by perfusion assessment after the ligation of the LAD by SPY and arteriograms.

Perfusion assessment

Tissue perfusion through the left ventricular wall was assessed with the SPY System (LifeCell Corporation, Branchburg, NJ, USA) prior and immediately after the occlusion of the LAD. Indocyanine green (IC-Green) fluorescent dye was injected intravenously followed by SPY scanning of the anterior left ventricular wall for the presence of the dye. Higher fluorescence intensity was interpreted as higher perfusion. The images collected prior to the LAD ligation and immediately after the ligation were used to determine the relative loss in perfusion, the precise location and size of ischemic area in the left ventricle (ischemia of the septum or the right ventricle are not visible in the field of view of the SPY).

Arteriograms

X-ray fluoroscopy (Advantx Systems Fluoroscope, GE Medical Systems, Wauwatosa, WI, USA) was utilized to view the coronary arteries of the heart prior to LAD occlusion, after the occlusion, and 2 and 7 weeks after MI. The femoral arteries were accessed via a cut-down followed by a modified Seldinger technique with a J-tipped angiographic guidewire (Bard Medical, Covington, GA, USA). A 6-French catheter (Boston Scientific, Marborough, MA, USA) was guided to the LAD. Arteries were visualized with injection of Isovue-300 iodine contrast agent (Bracco Diagnostics Inc., Monroe Township, NJ, USA) into the catheter. A TIMI score of 0, 1, 2 or 3 was assigned to assess flow through the LAD immediately after the occlusion, at 2 weeks and at 7 weeks. A score of 0 indicated no flow past occlusion site, a score of 1 indicates slow flow past the occlusion site but no filling of the distal regions of the arteries, a score of 2 indicates slow flow past the occlusion with filling of the distal arteries and a score of 3 indicates unimpeded flow.26 Arteriograms performed prior to occlusion and after 7 weeks were compared to assess angiogenesis at the macroscopic level. The number of animals with new vessels was recorded along with the vessel length and compared among different experimental groups.

Gene electrotransfer

GET, plasmid DNA injection only or sham injection of saline were administered following SPY perfusion assessment, within an hour of LAD occlusion. This time point was chosen to minimize stress to the animals by performing only one invasive, open-heart procedure and avoiding separate surgeries for LAD occlusion and GET treatment.

Four sites bordering ischemia were selected for GET. A 7-mm, four needle (5 mm electrode gap) penetrating electrode with an injection port was used for DNA delivery to the heart as previously described.20,21 For each treatment site, the electrode was placed on the surface of the myocardium, with electrode needles penetrating into the myocardium to 7 mm. A hypodermic needle was used to inject 100 μl of plasmid DNA encoding VEGF-A (pVEGF-A) in saline at 2 mg ml−1 centrally between the electrode needles through the injection port. The hypodermic needle was withdrawn to prevent electrical interference. An electric field was established between and around the electrode needles with applied voltage to accomplish electroporation and DNA delivery at the treatment site. Our custom-built pulse generator and software continuously captured the echocardiogram of the animal and enabled synchronizing pulsed with the R-wave of the echocardiogram (Accusync Medical Research Company, Milford, CT, USA). Each site was immediately pulsed eight times with 20-ms pulses at an amplitude of 60 V. Animals randomly assigned to the negative control groups received either saline injections or plasmid DNA injection without electrotransfer.

Immunofluorescence staining

Seven weeks after MI, animals were killed, and tissue samples were collected, fixed in 10% formalin, paraffin embedded and sectioned. Slides were stained for von Willebrand factor to identify microvasculature with a

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Blood Vessel Staining Kit (Millipore, Billerica, MA, USA). The number of vessels per field of view was counted and averaged over 10 randomly selected fields of view within each treatment region. Experimental groups were then compared based on the average number of vessels for vessel density.

**Echocardiography**

Cardiac ultrasound was performed with a Sonosite 180 Plus SonoHeart Elite Ultrasound (Sonosite Inc., Bothell, WA, USA)22,23 prior to the occlusion of the LAD, immediately after MI, 2 weeks after MI and 7 weeks after MI. Two-dimensional and M-mode echocardiography was used to measure left ventricular end-systolic diameter, left ventricular end-diastolic diameter, left ventricular wall thickness and intraventricular septum during systole and diastole. The Teicholz method was used to calculate left ventricular end-systolic volume, left ventricular end-diastolic volume, CO, fractional shortening, stroke volume and ejection fraction.26

**Enzyme-linked immunosorbent assays**

Blood samples were collected prior to MI, 24 h after occlusion and at 7 weeks for analysis of changes of cardiac protein levels. Plasma samples were tested for porcine cardiac troponin-I (Kamiya Biomedical, Seattle, WA, USA) and CK-MM (Kamiya Biomedical) according to the ELISA Kit manufacturer’s instructions for indications of initial myocardial damage and the presence of heart failure.

**Myocardial infarct size measurements**

At 7 weeks, hearts were harvested for infarct size assessment and histological analysis. Harvested hearts were sectioned into 1-cm coronal slices (perpendicular to the long axis of the heart). The slices were incubated with 1% triphenyltetrazolium (Sigma-Aldrich, St Louis, MO, USA) at 37 °C for 30 min. Each side of each slide was then photographed and the infarct size was measured using the ImageJ software (NIH, Bethesda, MD, USA) to trace the infarct in each image. In order to calculate the total volume of the infarct, the area measurement from each side of individual slices was averaged and multiplied by the thickness of the slice. The total infarct volume is the sum of individual infarct volumes from each heart slice.27 The total myocardial infarct volume included measurements from both ventricles and the intraventricular septum. Left ventricular MI surface area was also measured in order to compare the area of left ventricular ischemia induced by occlusion of the LAD to the area of MI after treatment of the left ventricle. ImageJ software was used to outline the perimeter of the MI on the pericardial surface of the left ventricle from each slice. The average from each side of the slice was multiplied by the thickness of the slice. The sum of areas from each slice was then used to determine the area of left ventricular MI.

**Conclusion**

End of study procedure

On the final day of the study, animals were anesthetized as follows. After induction of anesthesia by ketamine (20 mg kg⁻¹) and diazepam (3.5 mg kg⁻¹), pigs were anesthetized with isoflurane via a nose mask and intubated, with respiration maintained by a volume-controlled DRE Bonair anesthesia ventilator (DRE Medical Inc.). A final arteriogram was performed followed by sternotomy as described above. Following final assessment of perfusion by SPY procedure, animal was killed by an intracardiac injection of 5 ml of fatal plus.

**Statistical analyses**

All quantitative data with calculated means were compared using a one-way or two-way analysis of variance with P < 0.05 considered to be statistically significant. For analysis of variance yielding statistically significant differences, the Tukey’s multiple comparisons test was performed to determine which data sets were significantly different. All quantitative data were reported with the s.e.m. All proportions were compared with the chi-square test for proportions, with P < 0.05 considered significant.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.
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Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt)