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ORIGINAL ARTICLE

Increased perfusion and angiogenesis in a hindlimb ischemia model with plasmid FGF-2 delivered by noninvasive electroporation

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Gene therapy approaches delivering fibroblast growth factor-2 (FGF-2) have shown promise as a potential treatment for increasing blood flow to ischemic limbs. Currently, effective noninvasive techniques to deliver plasmids encoding genes of therapeutic interest, such as FGF-2, are limited. We sought to determine if intradermal injection of plasmid DNA encoding FGF-2 (pFGF) followed by noninvasive cutaneous electroporation (pFGFE+) could increase blood flow and angiogenesis in a rat model of hindlimb ischemia. pFGFE+ or control treatments were administered on postoperative day 0. Compared to injection of pFGF alone (pFGFE−), delivery of pFGFE+ significantly increased FGF-2 expression for 10 days. Further, the increase in FGF-2 expression with pFGFE+ was sufficient to significantly increase ischemic limb blood flow, measured by laser Doppler perfusion imaging, beginning on postoperative day 3. Ischemic limb blood flow in the pFGFE+ treatment group remained significantly higher than all control groups through the end point of the study, postoperative day 14. Immunohistochemical staining of gastrocnemius cross sections determined there was a twofold increase in capillary density in the pFGFE+ treatment group. Our results suggest that pFGFE+ is a potential noninvasive, nonviral therapeutic approach to increase perfusion and angiogenesis for the treatment of limb ischemia.

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Introduction

Peripheral artery disease (PAD), resulting from atherosclerosis, is one of the leading causes of morbidity and mortality in the western world.[1] The primary pathophysiology of PAD is reduction of blood flow to the lower extremities that commonly results in one of two clinical presentations, intermittent claudication (IC) or critical limb ischemia (CLI). IC is manifested by insufficient blood flow during exercise. The more severe manifestation, CLI, results from insufficient blood flow to the limb even when the effected limb is at rest. Current treatment options for PAD include risk factor reduction, physical therapy and training, and pharmacological treatment of the underlying atherosclerosis. Also, in patients with severe IC or CLI, interventional (catheter-based) or open surgical procedures may be used to revascularize the ischemic limb. However, direct revascularization is often impossible due to the anatomic extent of PAD and is also limited by associated comorbidities, such as diabetes, coronary artery disease or stroke, requiring a majority of patients to undergo limb amputation.[2–5] Currently, an effective pharmacological treatment to increase blood flow to ischemic limbs is not available.[6] The lack of an effective pharmacological treatment and the limits of interventional and surgical procedures have spurred an intense investigation of alternative approaches, such as therapeutic angiogenesis, for the treatment of PAD.

Angiogenesis has a major role in both health and disease. The formation of new blood vessels occurs during normal physiological processes, such as embryonic development and wound healing.[7] Insufficient neovascularization is characteristic of several diseases including ischemic heart and limb diseases, neurodegeneration and osteoporosis.[8] The initiation and progression of angiogenesis is mediated through the production of growth factors and cytokines in response to disease or injury. Therapeutic approaches delivering angiogenic growth factors, such as members of the vascular endothelial growth factor and fibroblast growth factor (FGF) families, are emerging as promising treatment options for IC and CLI in patients when direct revascularization is not possible.[9] FGFs have an integral role in angiogenesis by modulating multiple steps including degradation of the extracellular matrix and promotion of vascular endothelial cell proliferation, migration and morphogenesis.[10] There are currently greater than 20 known FGFs that share approximately 30–70% homology

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in their amino-acid sequences. Of the FGF family members, FGF-1 and FGF-2 have been the most intensely studied and most frequently used in emerging therapeutic angiogenesis approaches.

The overall aim of this study was to elucidate a noninvasive therapeutic approach for delivering FGF-2 as a potential treatment for IC and CLI. In previous studies, intramuscular injection of plasmid FGF-2,[12] alone or followed by intramuscular electroporation (EP),[13,14] increased angiogenesis and blood flow to ischemic limbs. In this study, we show that delivery of a plasmid encoding FGF-2 (pFGF) to the skin by intradermal injection and noninvasive EP (pFGFE+) significantly increases FGF-2 expression compared to injection of the plasmid without EP (pFGFE−). The increase in FGF-2 expression with pFGFE+ was sufficient to significantly increase blood flow and angiogenesis in a rat model of limb ischemia. The results presented here suggest that pFGFE+ is a novel potential noninvasive therapeutic angiogenesis approach for the treatment of PAD-related limb ischemia.

Results

FGF-2 expression kinetics

After determining pFGF transfection increased FGF-2 protein expression *in vitro* (data not shown), we then determined if pFGFE+ could increase FGF-2 protein expression *in vivo* (Figure 1). At the time points indicated in Figure 1, skin from the treated areas was excised and assayed for FGF-2 expression by enzyme-linked immunosorbent assay. For 10 days after treatment, pFGFE+ significantly increased FGF-2 protein levels, compared to pFGFE−, ($P<0.05$) before decreasing to background levels at days 14 and 17. The application of an electric field to tissues alone can transiently induce expression of some genes, including angiogenic growth factors,[15,16] but

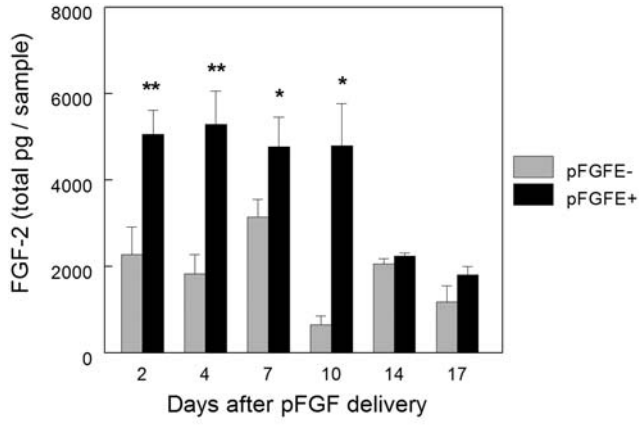

Figure 1 FGF-2 expression kinetics. At the indicated time points, skin samples were harvested from the delivery site and assayed for FGF-2 protein expression by enzyme-linked immunosorbent assay (ELISA). To determine FGF-2 expression resulting from pFGFE+ and pFGFE−, the average FGF-2 expression in untreated skin ($n=4$, 1416 ± 326 total pg per sample) was subtracted from the total pg per sample for each treatment site. Day 2, $n=10$; days 4, 7 and 10, $n=6$ and days 14 and 17, $n=8$ per group per time point. pFGFE+ = 300 V cm^{-1}, 150 ms. $^{**}P<0.01$, $^{*}P<0.05$. pFGFE−, intradermal injection of plasmid FGF-2; pFGFE+, intradermal injection of plasmid FGF-2 followed by electroporation.

injection of the vector backbone, lacking the FGF-2 cDNA insert, followed by EP (pVAXE+) resulted in similar levels of FGF-2 expression as untreated controls (P−E−) ($n=4$, 1416 ± 326 total pg per sample).

Plasmid FGF-2 with EP increases blood flow in the ischemic hindlimb

We next evaluated if pFGFE+ treatment could increase blood flow in a rat model of hindlimb ischemia. Immediately postoperatively (day 0) pFGFE+ or control treatments were administered at two sites on the medial aspect of the ischemic limb. A laser Doppler perfusion imager was used to measure perfusion in the distal area of both the ischemic and nonischemic limbs preoperatively (baseline), postoperatively but before treatment (day 0) and on postoperative days (PODs) 1, 3, 7 and 14 (Figure 2a). Immediately postoperatively, blood flow decreased to approximately 40% of baseline indicating the hindlimb was effectively rendered ischemic. In Figure 2b blood flow is reported for each treatment group as the ratio of blood flow in the ischemic hindlimb to the nonischemic hindlimb (I/NI) (Figure 2b, top panel) and as a percent of the perfusion recorded at baseline (Figure 2b, bottom panel). There was a significant difference in limb blood flow beginning on POD 3 between all treatment groups for I/NI ($P<0.02$) and as a percentage of baseline levels ($P<0.001$). Also on POD 3, blood flow in the pFGFE+ treatment group was significantly greater than all of the control groups ($P<0.05$). Blood flow in the ischemic limb continued to be higher in the pFGFE+ treatment group, compared to control treatment groups, at all subsequent time points in the study ($P<0.05$, all time points for percent of baseline blood flow and I/NI). The pFGFE− treatment group showed a slight, but not significant, increase in blood flow compared to the pVAXE+ and P−E− treatment groups on PODs 7 and 14.

Plasmid FGF-2 with EP increases angiogenesis in the ischemic hindlimb

Next, we determined if the increase in limb perfusion in the pFGFE+ treatment group resulted from an increase in angiogenesis. In rodent models of hindlimb ischemia, as well as in patients with PAD, angiogenesis typically occurs in the gastrocnemius muscle, or distal to the arterial occlusion.[17] Thus, on POD 14 samples were harvested from the gastrocnemius muscle of the ischemic limb and capillary density visualized by immunohistochemical staining for the endothelial cell marker factor-VIII-associated antigen. Representative fields ($\times400$) from each of the four treatment groups are shown in Figure 3a. In Figure 3b, the average number of vessels in five randomly selected high-power fields ($\times400$) for five animals in each treatment group is reported. There was an approximately twofold increase in the number of capillaries in the pFGFE+ treatment group, compared to all control groups ($P<0.001$), indicating the increase in blood flow to the ischemic limb with pFGFE+ was in fact due to an increase in angiogenesis.

Discussion

Direct injection of naked plasmid DNA has been intensively investigated as a gene delivery approach for

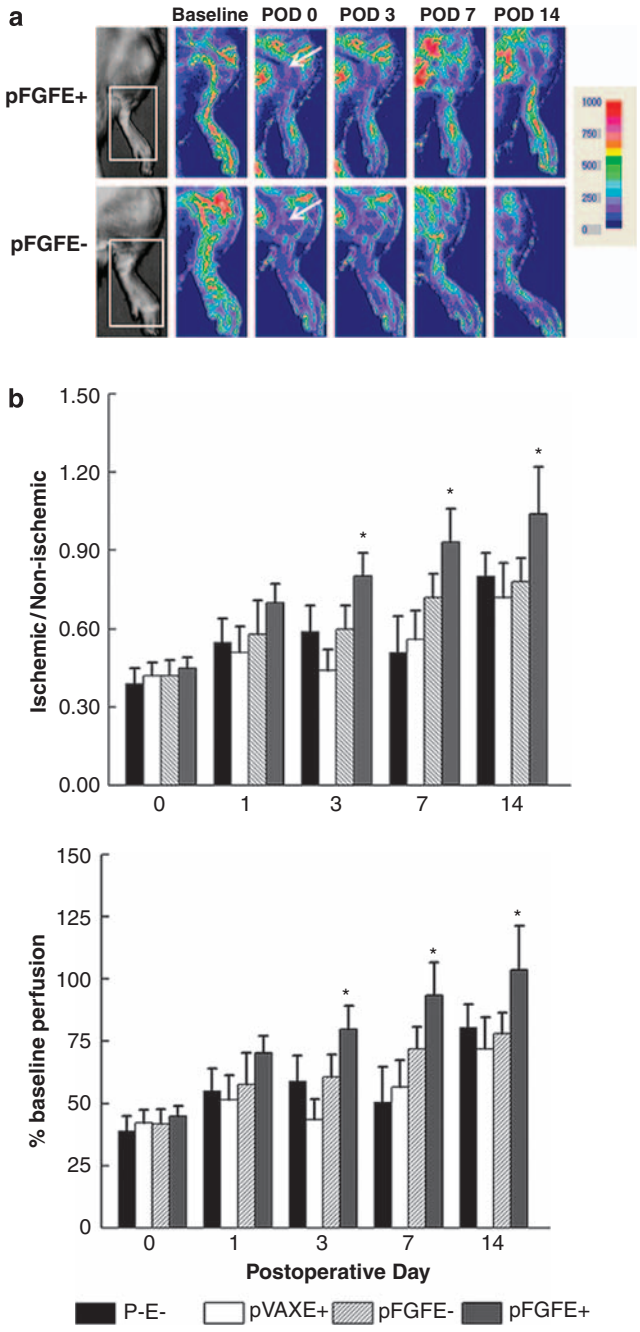

Figure 2 Effect of pFGFE+ on ischemic limb blood flow. (**a**) Representative laser Doppler perfusion images for the pFGFE+ and pFGFE− treatment groups from baseline to day 14 postoperatively. The white box indicates the approximate area where blood flow was assessed. The arrows indicate the absence of perfusion in the area of the femoral artery after the operation to induce hindlimb ischemia. (**b**) Quantification of blood flow as determined by laser Doppler perfusion imaging. (Top panel) Ratio of blood flow in the ischemic limb to the nonischemic limb (I/NI). (Bottom panel) Postoperative blood flow as a percentage of baseline line blood flow. $n=6$ per group per time point. *$P<0.05$ compared to all controls. pFGFE+, intradermal injection of plasmid FGF-2 followed by electroporation; pFGFE−, intradermal injection of plasmid FGF-2; pVAXE+, intradermal injection of vector backbone followed by electroporation; P−E−, no treatment.

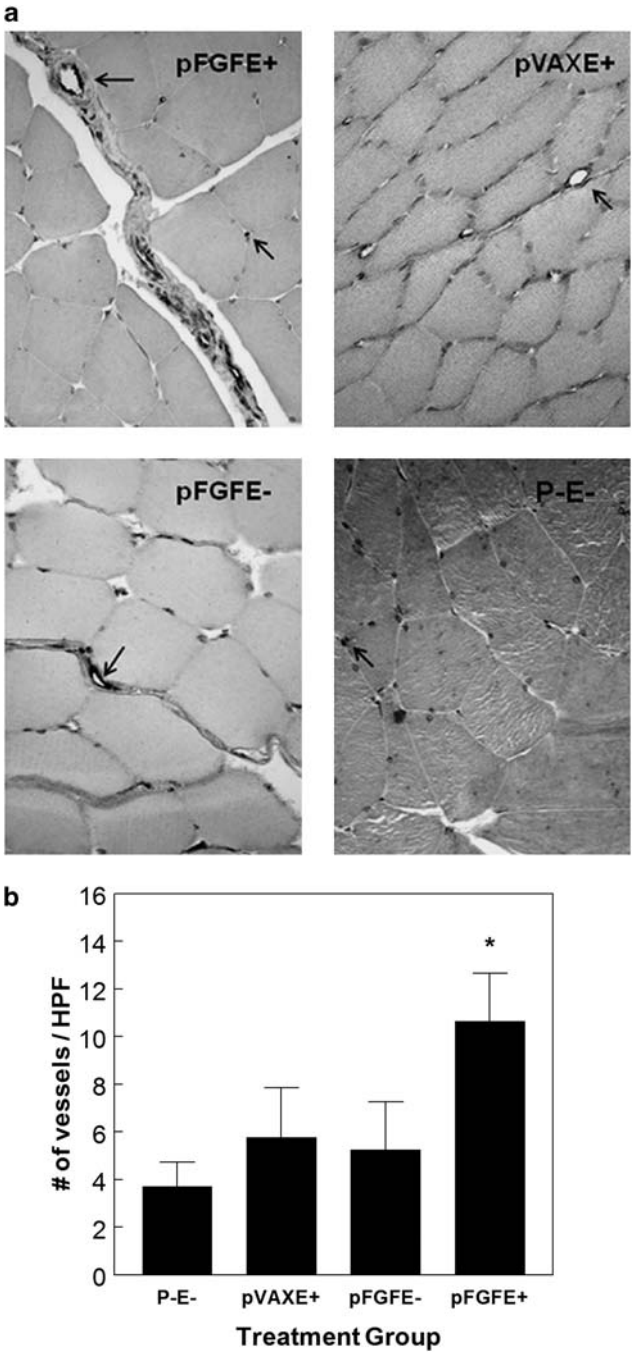

Figure 3 Effect of pFGFE+ on neovascularization. (**a**) Representative cross sections (×400) of factor VIII-associated antigen immunohistological staining of cross sections excised from the gastrocnemius on POD 14. Arrows indicate examples of stained vessels. (**b**) Quantification of capillary density. The average number of capillaries in five fields, for five animals in each treatment group. *$P<0.01$ compared to all controls. HPF, high-power field (×400). pFGFE+, intradermal injection of plasmid FGF-2 followed by electroporation; pFGFE−, intradermal injection of plasmid FGF-2; pVAXE+, intradermal injection of vector backbone followed by electroporation; P−E−, no treatment.

the treatment of a variety of diseases. One drawback to this approach is inefficient uptake of the plasmid by cells resulting in low levels of gene expression.[18] *In vivo* EP has been successfully used to increase the uptake and expression of naked plasmid DNA in the skin and other target tissues. The skin is an attractive target for the

delivery of plasmid DNA because it allows for enhanced control over expression levels and aids in targeting expression to specific tissue areas. Further, if higher expression levels are needed, the area treated or number of treatments can be increased. Plasmid DNA has been successfully delivered to the skin with *in vivo* EP for both systemic and tissue-specific expression.[19–22] This approach has shown promise in several fields including vaccines[22–26] and wound healing.[27–30]

The design of the electrode used for plasmid delivery in this study, multielectrode array,[11] allows for this potential treatment for limb ischemia to be readily translated to a clinical setting. One drawback of other electrode designs is delivery of plasmid DNA to a large surface area requires the distance between the electrode pairs to be increased thereby the applied voltage must also be increased for cell permeabilization to be effective. The design of the multielectrode array allows for its dimensions to be expanded by incorporating a larger number of electrode pairs thus enabling a larger surface area to be treated without the need to increase the distance between electrodes.

In addition to direct injection of naked plasmid DNA, the most prevalent methods of therapeutic administration of FGF-2 in the literature are viral-mediated gene transfer and recombinant protein delivery. These methods are promising for treatment of PAD but have drawbacks. In human clinical trials, viral vectors have caused inflammatory responses, formation of antibodies to the viruses, transient fever[31] and hepatotoxicity.[32] Also, relative to nonviral gene transfer approaches, viral-mediated delivery results in long-term and high levels of gene expression increasing the potential for the occurrence of the adverse side effects associated with high levels of FGF-2 expression, such as toxicity and proteinuria.[6,33] In both animal models[34–36] and clinical trials,[37–39] intramuscular injection or intra-arterial administration of recombinant FGF-2 protein improved PAD-related symptoms and or blood flow to the ischemia limb, but the use of recombinant protein in a clinical setting is often not practical due to its short half-life,[37,40] poor bioavailability and consequently frequent administration is often required to sustain lasting effects. The strategy developed here to deliver FGF-2 to an ischemic limb is an attractive alternative to viral and recombinant protein approaches because it allows for control of localized expression level and duration. Also, pFGFE+ circumvents the possibility of adverse side effects and practicality issues associated with viral and recombinant protein delivery approaches.

Other studies have shown that intramuscular injection of plasmid FGF-2 alone[11] or followed by intramuscular EP[13,14] can improve ischemic hindlimb blood flow and increase angiogenesis. Here, we have presented a simple, noninvasive approach for delivery of pFGF with EP that effectively increases FGF-2 protein expression, ischemic limb blood flow and angiogenesis. The increased blood flow and angiogenesis with pFGFE+ treatment may be occurring through several known *in vivo* mechanisms. FGF-2 is normally confined to the cell compartment but is exported from the cell during active angiogenesis to exert downstream effects.[41] Thus, although pFGFE+ treatment is administered to the skin, increased levels of FGF-2 protein after treatment and subsequent export could activate downstream angiogenic pathways. For example, a recent study by Fujii *et al*.[12] showed that in a model of hindlimb ischemia, intramuscular injection of a pFGF increased the expression of placental growth factor through the upregulation of vascular endothelial growth factor expression. Placental growth factor alone is known to increase angiogenesis in models of hindlimb ischemia, and it is possible a similar mechanism may be occurring with pFGFE+.[42,43] Another possible mechanism for the increase in angiogenesis and hindlimb blood flow with pFGFE+ treatment is the upregulation of hepatocyte growth factor. FGF-2 stimulates hepatocyte growth factor expression, which itself has also been successful in increasing perfusion in hindlimb ischemia models.[44,45] Further studies to characterize the exact mechanism of pFGFE+ mediated angiogenesis are currently underway.

In summary, pFGFE+ treatment significantly increased FGF-2 expression for 10 days after delivery and this increase was sufficient to significantly increase blood flow and angiogenesis in a rat model of hindlimb ischemia. Translation of this therapeutic approach to an appropriate large animal model, and eventually to the clinic, will be the focus of future studies. Overall, EP-mediated intradermal delivery of plasmid FGF-2 is a potential noninvasive, nonviral therapeutic approach to increase perfusion and angiogenesis in ischemic limbs and warrants further investigation as a possible treatment for PAD.

Materials and methods

Experimental animals

All procedures were approved by the Animal Use and Care Committee of the University of South Florida College of Medicine. A total of 34 male Sprague–Dawley rats (275–300 g) were used in this study; 10 rats were use to evaluate FGF-2 expression kinetics and 24 rats were used for analysis of perfusion and capillary density. Operations were performed using standard aseptic techniques. Before all procedures, animals were anesthetized with 2.5–3.0% isoflurane in oxygen.

Hindlimb ischemia model

Hindlimb ischemia was induced in the right hindlimb of the Sprague–Dawley rats. Through an approximately 2-cm-long incision parallel to the inguinal ligament, the saphenous artery was ligated distally of the bifurcation of the femoral artery into the saphenous and popliteal arteries. The most distal end of the external iliac was ligated immediately adjacent to the inguinal ligament and the femoral artery was ligated proximal and distal to the bifurcation of the superficial epigastric artery and vein. The superficial epigastric artery and vein were also ligated at two sites adjacent to the femoral bifurcation and cut between these sites. The femoral artery was then cut between the ligations adjacent to the inguinal ligament and distally of the superficial epigastric artery and vein. The femoral artery was then dissected free from the point of the distal ligation of the saphenous artery to the ligation of the femoral placed distally to the bifurcation of the superficial artery and vein.

FGF-2 plasmid construction

The CMV promoter was removed from the pVAX1 plasmid (Invitrogen, Carlsbad, CA, USA) to create a

promoterless pVAX1. The human FGF-2 plasmid (pFGF) was cloned by subcloning the hEF1-IF4g-hbFGF sequence from pBLAST45-hbFGF2 (Invivogen, San Diego, CA, USA) into the promoterless backbone of pVAX1. Where indicated, vector backbone controls are the pVAX1 backbone containing the hEF1-IF4g promoter.

Electroporation

After the operation to induce hindlimb, we randomized ischemia animals into one of four treatment groups: injection of pFGF with EP (pFGFE+), injection of pFGF without EP (pFGFE−), injection of vector backbone with EP (pVAXE+) and no treatment (P−E−). All injections were performed using a 25-gauge, 5/8-inch-length needle. Animals received two 50 μl intradermal injections of plasmid DNA (2 mg ml^{-1}) in sterile injectable saline. The multielectrode array [11] was then placed over the injection bubble and 150 ms pulses applied at a field strength of 300 V cm^{-1} (delivery to skin of rat flank for initial expression kinetics) or 250 V cm^{-1} (delivery to the hindlimb). FGF-2 expression levels resulting from pFGFE+ and pFGFE− treatments were equivalent at 300 V cm^{-1} when delivered to skin on flank of a rat and 250 V cm^{-1} for delivery to the skin of the hindlimb and did not differ between ischemic and nonischemic limbs (data not shown).

FGF-2 protein expression

FGF-2 protein expression was determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). At the indicated time points, the delivery sites were excised and snap frozen on dry ice. Tissue samples were homogenized in 1–2 ml of lysis buffer (150 mmol l^{-1} NaCl, 20 mmol l^{-1} sodium phosphate (pH 7.4), 10% glycerol, 1% NP-40 and protease inhibitors (Roche, Indianapolis, IN, USA)). Homogenates were then centrifuged at 4 °C for 10 min at 10 000 r.p.m. The supernatants were removed and assayed for FGF-2 expression according to manufacturer's instructions.

Measurement of blood flow by laser Doppler perfusion imaging

A laser Doppler perfusion imager (Moor Instruments Ltd., Devon, UK) was used to measure hindlimb blood flow. Before measuring perfusion, we anesthetized and placed animals on a warming pad to ensure constant body temperature. A low-intensity (2 mW) laser light beam ($\lambda = 632.8$ nm) scanned the surface of the skin without contact at a standardized working distance of 25 cm. Scan modus was set at 10 ms per pixel and resolution at 256×256 pixels. Three scans were completed per time point for each animal for both the ischemic and nonischemic limbs and average perfusion in arbitrary units (flux) was determined separately for each limb. Perfusion in the ischemic limb was normalized to the contralateral nonischemic limb (I/NI) to minimize variation due to ambient light and temperature. Baseline perfusion was assessed immediately preoperatively and postoperative perfusion assessed immediately after surgery but before treatment. The I/NI was used to calculate percentage of baseline perfusion at the postoperative time points.

Immunohistochemistry

Two weeks after treatment, animals were killed and tissue samples from the gastrocnemius muscle were excised and fixed in 10% neutral buffered formalin for at least 12 h at room temperature before embedding in paraffin and sectioning (4 μm). Capillaries in the cross sections were visualized using the Blood Vessel Staining Kit (Millipore, Billerica, MA, USA) according to manufacturer's instructions for factor VIII with hematoxylin counterstaining. The number of factor VIII-positive vessels was manually counted in a blinded manner in five randomly selected fields ($\times 400$) for five animals from each treatment group. The stained sections were analyzed using a standard light microscope (Olympus BX51; Olympus, Center Valley, PA, USA). The average capillary density per high-power field ($\times 400$) for each treatment group is reported. Representative fields from each treatment group were photographed at $\times 400$ with a Spot Insight 2 digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Statistical analysis

All values are reported as the mean ± s.e.m. unless otherwise noted. Analysis of FGF-2 expression and capillary density was completed using a two-tailed Student's *t*-test. Analysis of blood flow was completed by analysis of variance with *post hoc* Fisher's least significant difference to adjust for multiple group comparisons. A *post hoc* Bonferroni correction was used to adjust for multiple comparisons to pFGFE+ for analysis of capillary density. All statistical analyses were completed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA).

Conflict of interest

Richard Heller is an inventor on patents used in this work. These patents have been licensed to RMR Technologies, LLC and Inovio Biomedical Corporation. Richard Heller has ownership interest in RMR Technologies and owns stock and stock options of Inovio Biomedical Corporation.

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