Published in final edited form as:

Biomaterials. 2016 September ; 102: 9–19. doi:10.1016/j.biomaterials.2016.06.011.

Therapeutic assessment of mesenchymal stem cells delivered within a PEGylated fibrin gel following an ischemic injury

Laura M. Ricles, PhD¹, Pei-Ling Hsieh, MS², Nicholas Dana¹, Viktoriya Rybalko, PhD¹, Chelsea Kraynak¹, Roger P. Farrar, PhD², and Laura J. Suggs, PhD¹

¹Department of Biomedical Engineering, The University of Texas at Austin, Austin, TX, USA, 78712

²Department of Kinesiology and Health Education, The University of Texas at Austin, Austin, TX, USA, 78712

Abstract

Corresponding author: Laura J. Suggs.

Contact information:

Laura M. Ricles, Phone: 512-232-8593, Fax: 512-471-0616, Mailing address: 107 W. Dean Keeton St., C0800, Austin, TX 78712, laura.ricles@utexas.edu

Pei-Ling Hsieh, Phone: 512-471-8621, Fax: 512-471-0946, Mailing address: 2109 San Jacinto Blvd., D3700, Austin, TX 78712, katiehsieh@utexas.edu

Nicholas Dana, Phone: 512-232-8593, Fax: 512-471-0616, Mailing address: 107 W. Dean Keeton St., C0800, Austin, TX 78712, n.dana@utexas.edu

Nicholas Dana, Phone: 512-232-8593, Fax: 512-471-0616, Mailing address: 107 W. Dean Keeton St., C0800, Austin, TX 78712, n.dana@utexas.edu

Viktoriya Rybalko, Phone: 512-232-8593, Fax: 512-471-0616, Mailing address: 107 W. Dean Keeton St., C0800, Austin, TX 78712, viktoriya.rybalko@gmail.com

Chelsea Kraynak, Phone: 512-232-8593, Fax: 512-471-0616, Mailing address: 107 W. Dean Keeton St., C0800, Austin, TX 78712, chelsea.kraynak@gmail.com

Roger P. Farrar, Phone: 512-471-8621, Fax: 512-471-0946, Mailing address: 2109 San Jacinto Blvd., D3700, Austin, TX 78712, rfarrar@austin.utexas.edu

Laura J. Suggs, Phone: 512-232-1671, Fax: 512-471-0616, Mailing address: 107 W. Dean Keeton St., C0800, Austin, TX 78712, laura.suggs@mail.utexas.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Disclosure Statement

The authors have no disclosures.

Supplemental Information

Syringe viability study

Cell viability following injection within PEGylated fibrin gels was evaluated using a LIVE/DEAD stain. PEGylated fibrin injections were prepared by combining difunctional succinimidyl glutarate PEG (4 mg/mL in PBS without calcium; NOF America) with fibrinogen (40 mg/mL in PBS without calcium; Sigma) in a 1:1 volume ratio. An equal volume of rat MSCs (passage 5) was mixed with the PEGylated fibrin solution in a 1:1 volume ratio at a concentration of 2×10^5 cells/mL. The solution was then loaded into a 23G needle syringe, followed by an equal volume of thrombin (25 U/mL in 40 mM CaCl₂). The solution was mixed thoroughly within the syringe and the gel solution (500 μ L) was injected into a 24 well plate. The final concentrations in the gel were 5 mg/mL of fibrinogen; 0.5 mg/mL of SG-PEG-SG; 5×10^5 cells/mL; and 12.5 U/mL of thrombin. A gel which was formed in the well plate and not injected through a syringe was used as a positive control. The gels were stored at 37°C in MSC growth media. Twenty four hours following the injection, the cells were stained with a LIVE/DEAD stain to assess cell viability. Briefly, the media was removed and the gels were washed with PBS for 15 minutes. The PBS washes were repeated for a total of 90 minutes. The gels were then incubated in a LIVE/DEAD stain solution consisting of 10 μ M calcein and 10 μ M ethidium homodimer for 1 hour at 37°C. The stain was removed and the gels were washed with PBS, fixed in 10% formalin for 20 minutes, washed with PBS, and imaged on a Leica DMI2000B microscope equipped with a Leica DFC290 camera. Gels incubated with methanol prior to staining were used as a dead control condition.

The intent of the current study was to investigate the therapeutic contribution of MSCs to vascular regeneration and functional recovery of ischemic tissue. We used a rodent hind limb ischemia model and intramuscularly delivered MSCs within a PEGylated fibrin gel matrix. Within this model, we demonstrated that MSC therapy, when delivered in PEGylated fibrin, results in significantly higher mature blood vessel formation, which allows for greater functional recovery of skeletal muscle tissue as assessed using force production measurements. We observed initial signs of vascular repair at early time points when MSCs were delivered without PEGylated fibrin, but this did not persist or lead to recovery of the tissue in the long-term. Furthermore, animals which were treated with PEGylated fibrin alone exhibited a greater number of mature blood vessels, but they did not arterialize and did not show improvements in force production. These results demonstrate that revascularization of ischemic tissue may be a necessary but not sufficient step to complete functional repair of the injured tissue. This work has implications on stem cell therapies for ischemic diseases and also potentially on how such therapies are evaluated.

Introduction

Peripheral artery disease (PAD) is a growing health concern in the United States, with 8–10 million people being affected by the disease.[1] Intermittent claudication is an early, moderate manifestation of the disease and is characterized by leg pain and muscle weakness. [1, 2] However, PAD can quickly develop into critical limb ischemia, which is a more chronic and severe problem.[1, 2] Critical limb ischemia often results in tissue infection, death, and potentially amputation because the resting metabolic needs of the tissue are not met by the available blood supply.[1, 2] Although current revascularization therapies (including bypass surgery and balloon angioplasty) may provide some benefit to patients, not all patients are eligible for such procedures.[1–4] Major limb amputation is often necessary if revascularization therapy is not possible, or was not successful.[3, 4]

Many have investigated the use of angiogenic gene and protein therapy as an alternative revascularization strategy.[2, 5, 6] Although gene and protein strategies have shown some success in preclinical animal models, there have not been significant improvements in clinical studies.[5, 6] This could potentially be attributed to the fact that the ischemic wound healing response is very complex, and thus delivering the correct genes/proteins is very difficult,[5, 6] especially when taking into account necessary temporal release kinetics and concentration gradients. As a result, cell-based approaches are an attractive therapy option because the cells can supply the necessary cytokines and growth factors necessary to promote, as well as support, vascular regeneration.[2] In addition, the use of multipotent stem cells, such as bone marrow-derived mesenchymal stem cells (MSCs), could provide other therapeutic effects in addition to blood vessel growth.[2]

Mesenchymal stem cells (MSCs) are an adult stem cell population found within multiple regions of the body[7, 8] which can terminally differentiate into mesodermal lineages. MSCs have been shown to secrete factors which are pro-angiogenic and promote wound healing, and thus are an ideal candidate cell type for therapeutic revascularization.[7, 9–11] Many research groups have demonstrated blood vessel formation and increased reperfusion of ischemic areas following delivery of MSCs or other stem cells,[2, 11, 12] either through

direct cell differentiation or paracrine effects[7, 10, 11]. A gap exists in the literature between demonstrating increased perfusion as a result of stem cell treatment and whether or not that leads to functional recovery.

This study evaluated the use of bone marrow-derived mesenchymal stem cells as a therapy for peripheral arterial disease. A rat model of acute hind limb ischemia was used in which the femoral artery was ligated and excised. MSCs were intramuscularly delivered within a PEGylated fibrin gel following ischemia. The PEGylated fibrin delivery system offers several advantages, including retaining MSCs at the delivery site. In addition, fibrin is a natural component of the wound healing response and has been demonstrated to have angiogenic properties.[13, 14] To evaluate the therapeutic contribution of treatment, restoration of blood flow and muscle function were evaluated. Histological analysis was also performed to evaluate tissue architecture and blood vessel formation. The results of this study have important implications pertaining to alternative cell-based therapies for ischemic and cardiovascular diseases.

Materials and Methods

Rat MSC isolation and culture

Bone marrow MSCs were isolated from Lewis rats (8–10 weeks old). The femoral marrow cavity was flushed and adherent cells were collected and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% glutamax, and 1% penicillin-streptomycin. After 24 hours, non-adherent cells were removed by replacing the medium. The results adherent cells underwent medium changes every 2 days and were passaged once they reached approximately 80% confluency. Passage 4 cells were used in the current study.

Ischemic injury

Animal handling and care followed the recommendations of the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. All protocols were approved by the Animal Care Committee at the University of Texas at Austin (AUP-2011-00162). Lewis rats (11 weeks) weighing 250–300 g were used. To induce hind limb ischemia, femoral artery ligation in Lewis rats (11 weeks, male) was performed. Rats were anesthetized using isoflurane (0.5–2%) infused with oxygen (2 L/min). Through a small incision on the medial side of the thigh, the femoral artery of a single hind limb was separated from the nearby nerve and vein and ligated immediately distal to the inferior epigastric artery and proximal to the branch point of the popliteal and saphenous arteries using Prolene 5-0 sutures (Figure 1). The ligated segment (~0.5 cm) was then excised and the skin incision closed with interrupted sutures. The animal was allowed to recover overnight and the following day (about 24 hours later) therapy was delivered.

PEGylated fibrin gel delivery of MSCs

MSCs were injected intramuscularly into the gastrocnemius muscle of the ligated limb. PEGylated fibrin injections were prepared by combining difunctional succinimidyl glutarate PEG (4 mg/mL in PBS without calcium; NOF America) with human fibrinogen (40 mg/mL

in PBS without calcium; Sigma) in a 1:1 volume ratio. An equal volume of rat MSCs was mixed with the PEGylated fibrin solution in a 1:1 volume ratio at a concentration of 13×10^6 cells/mL. The solution was then loaded into a 23G needle syringe, followed by an equal volume of thrombin (25 U/mL in 40 mM CaCl_2). The solution was mixed thoroughly within the syringe and the gel solution (300 μL) was injected into the gastrocnemius of the rat. The final concentrations in the gel were 5 mg/mL of fibrinogen; 0.5 mg/mL of SG-PEG-SG; 3.33×10^6 cells/mL; and 12.5 U/mL of thrombin. Other treatment groups consisted of no treatment, PEGylated fibrin gel, MSCs, and 10% FBS containing DMEM (serum). For the PEGylated fibrin gel treatment, 300 μL of PEGylated fibrin gel was injected into the lateral gastrocnemius muscle, with the cell portion replaced by 10% FBS containing DMEM. For the MSC treatment, 300 μL of MSCs (3.33×10^6 cells/mL) suspended in 10% FBS containing DMEM was injected into the lateral gastrocnemius muscle. Prior to delivery, MSCs were fluorescently labeled with CellTracker™ CM-DiI (Invitrogen). The cells were incubated with CM-DiI (15 μM) at 37°C for 8 minutes and then 4°C for 15 minutes, washed with PBS, and resuspended in DMEM. For the serum group, 300 μL of 10% FBS containing DMEM was injected into the lateral gastrocnemius. The serum group (sham treatment) was included to evaluate if the injection procedure itself lead to a response (e.g. inflammatory response) that could contribute to vascular and/or tissue repair.

Blood flow measurements

The blood flow to the ischemic hind limbs was imaged using laser speckle imaging. Rats were anesthetized using isoflurane (0.5–2%) infused with oxygen (2 L/min). The speckle imaging system consisted of a diode laser (785 nm, 50 milliwatt; Thor Labs), Basler 1920 $\times$ 1080 monochrome CCD with a zoom lens (Zoom7000; Navitar) mounted on a microscope boom stand and used to record speckle images of blood perfusion. The raw speckle images were converted into speckle contrast images and analyzed using Matlab code to quantify the blood flow to the ischemic hind limb as a percentage of the contralateral control.

Force production measurements

Force production measurements of the lateral gastrocnemius muscle were conducted on all treatment groups with sample size of 5. The gastrocnemius muscle was surgically isolated from the surrounding tissue and innervation to the medial gastrocnemius muscle was removed. The Achilles tendon was secured to the lever arm of a dual-mode servomotor (Aurora Scientific Model 310B). The muscle was stimulated using a stimulator (A-M Systems Model 2100) with electrodes applied to the tibial nerve. The optimal length, or the length which produced the maximal twitch force, was determined by stimulating the muscle at 0.5 Hz. The maximal peak tetanic tension was measured by stimulating the muscle at the optimal length at a frequency of 150 Hz and the minimal voltage required to elicit a maximal response. Between each contraction there was a 2 minute period of rest. The temperature of the muscle was maintained with a heating lamp throughout the duration of the study. At the end of the study, the animal was sacrificed and the lateral gastrocnemius muscle was isolated. The muscle mass and muscle length were measured in order to calculate the cross sectional area (Equation 1), where Q is the fiber angle from the mid-line (19°). The fiber length is calculated by multiplying the muscle length by 0.38 (fiber length/muscle length ratio for the gastrocnemius), and the density of skeletal muscle is 1.056 g/cm³. The specific

tension was calculated using Equation 2. The specific tension was normalized to the contralateral control (% of contralateral control).

$$\text{Cross sectional area} = \frac{\text{muscle mass (mg)} * \cos Q}{\text{fiber length (mm)} * \text{density } (\frac{g}{cm^3})} \quad (1)$$

$$\text{Specific tension} = \frac{\text{maximal peak tetanic tension (N)}}{\text{cross sectional area (cm}^2\text{)}} \quad (2)$$

Histology

At the terminal endpoint of the study, animals were sacrificed and the gastrocnemius muscles were isolated, embedded in optimal cutting temperature (OCT) compound, submerged in liquid nitrogen-cooled isopentane, and stored at -80°C until further processing. The samples were cut into $12\text{ }\mu\text{m}$ thick sections using a cryostat and placed onto positively charged microscope slides. The tissue sections were fixed in 10% formalin for 15 minutes. Hematoxylin and eosin (H&E) and immunohistochemical and immunofluorescence staining were subsequently performed.

For H&E staining, the slides were fixed in 10% formalin for 15 minutes, followed by rinsing with PBS. The sections were then incubated in Mayer's hematoxylin solution (Electron Microscopy Sciences) for 15 minutes and rinsed in running tap water for 15 minutes. The sections were then dipped in Scott's bluing solution for 30 seconds, rinsed in distilled water for 30 seconds, and incubated in 95% ethanol for 30 seconds. The samples were then transferred into Eosin Y solution (Sigma-Aldrich) for 1 minute followed by serial washes in 95% ethanol, 100% ethanol, and xylene for 2 minutes each. The slides were then mounted with Cytoseal Mounting Media (Thermo Scientific) and viewed with a DMI2000B Leica microscope.

TUNEL staining was performed with the APO-BrdU TUNEL Assay Kit (Invitrogen). The slides were incubated in 0.125% trypsin for 20 minutes at 37°C , followed by permeabilization in 0.5% Triton X-100 for 30 minutes. The slides were then incubated with TdT and BrdUTP for 2 hours at 37°C . Alexa Fluor 488 dye-labeled anti-BrdU antibody was applied for 30 minutes. The slides were then counterstained with DAPI ($5\text{ }\mu\text{g/mL}$) for 15 minutes, mounted, and viewed using a fluorescence microscope (DMI2000B; Leica).

For immunohistochemistry, the slides were fixed in 10% formalin for 15 minutes, followed by rinsing with PBS. The sections were then incubated in 0.125% trypsin solution for 20 minutes at 37°C . They were then incubated in 0.3% hydrogen peroxide solution for 5 minutes, permeabilized in 0.5% Triton X-100 in TBST for 30 minutes, and blocked in 2.5% horse serum for 1 hour. The slides were incubated overnight at 4°C in the primary antibody (1:100 dilution in 2.5% horse serum) (SMA; Abcam). Following overnight incubation, the sections were incubated with ImmPRESS (rat absorbed mouse IgG) solution (Vector Laboratories) for 30 minutes and then ImmPACT DAB peroxidase substrate (Vector

Laboratories) for 5 minutes. The sections were counterstained with hematoxylin QS (Vector Laboratories), rinsed with running tap water, incubated in serial concentration of ethanol (95% and 100%) and xylene for 2 minutes each, and mounted. The SMA positive area and the number of SMA positive vessels were quantified using ImageJ.

For immunofluorescence staining, the slides were fixed in 10% formalin for 5 minutes, followed by rinsing with PBS. The sections were then blocked in 10% normal goat serum for 1 hour and incubated overnight at 4°C in the antibody (1:200 dilution in 2.5% goat serum) (lectin from *Bandeiraea simplicifolia*, FITC conjugated; Sigma Aldrich). The sections were then washed with PBS, counterstained with DAPI (5 µg/mL) for 15 minutes, mounted, and viewed using a fluorescence microscope (DMI2000B; Leica). The number of lectin positive cells were quantified using a grid system overlaid on the microscope images. The lectin positive cells associated with a cell nucleus and located within the overlaid grid were counted and normalized by the number of muscle fibers.

Combined ultrasound/photoacoustic imaging

A longitudinal photoacoustic imaging study using the rodent hind limb ischemia was done to compare photoacoustic signal generated gold nanoparticle (AuNP) labeled MSCs at day 0, 7, and 14. Spherical AuNPs (20 nm diameter) were synthesized as described previously [15], although they were not coated in polyethylene glycol. The AuNPs were centrifuged at 5000 RCF for 20 minutes, resuspended in deionized water, sterilized by UV for 30 minutes, centrifuged at 5000 RCF, and then resuspended in phenol-free DMEM media with 10% MSC-qualified FBS at 10^{12} AuNP/mL. Rat MSCs were plated at 5000 cells/cm² and incubated with 0.2 mL/cm² AuNP media for 24 hours. The AuNP media was then removed and the MSCs were washed twice with PBS and incubated with trypsin with 0.25% EDTA for 5 minutes (ATCC® 30-2101, American Type Culture Collection, VA, USA). Cells were then collected, centrifuged at 600 RCF to form a pellet, and resuspended in phenol-free DMEM media. To verify AuNP uptake by MSCs, 100 µL of cell suspension was placed in a 96-well plate and optical absorbance measurements were taken from 400 nm to 950 nm, in 2 nm increments, using a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader, BioTek Instruments, Inc., Vermont, USA).

One day prior to injection, femoral artery ligation was performed on two Lewis rats, as described above. The following day, intramuscular injection of AuNP labeled MSCs into the lateral gastrocnemius of the ligated limb, followed by photoacoustic imaging, was performed. Prior to injection, the lateral side of the ligated hind limb was shaved and depilatory lotion was used to remove remaining fur. One rat was treated with a 300 µL injection of 3.33×10^6 MSCs/mL mixed with the PEGylated fibrin gel, while the second rat was treated with a 300 µL injection of 3.33×10^6 MSCs/mL suspended in 10% FBS containing DMEM.

Photoacoustic (PA) and ultrasound (US) imaging took place immediately following injection. Following injection, sedation with isoflurane was continued and animal body temperature and respiration were monitored throughout imaging, in accordance with IACUC protocols. Optically transparent acoustic gel was used to acoustically couple the US/PA system and the rat hind limb. Imaging was done using a 21 MHz combined US/PA

transducer attached to a translational motor-stage for three dimensional volumetric imaging (Vevo 2100 LAZR®, Visual Sonics Inc., Toronto, Canada). PA images (spatial resolution $45 \times 37 \mu\text{m}^2$, lateral $\times$ axial, respectively) were acquired at 740 nm, which allowed for easy visualization of AuNP labeled MSCs, with reduced background signal from tissue chromophores, such as hemoglobin and melanin. Co-registered US images were also acquired that visualized the gastrocnemius muscle, as well as the tibia and fibula. The injection region was located in the PA field-of-view (FOV) and volumetric data were acquired in the center region by translating the transducer ($152 \mu\text{m}$ steps) orthogonal to the imaging plane and acquiring a two-dimensional PA and US image at each position. Following imaging a mark was placed on the animal's skin to identify the center of the imaging FOV. Imaging studies were done on both rats following initial injection (day zero) and repeated on day 7 and day 14 following injection, after which animals were sacrificed according to IACUC protocols.

Upon completion of all imaging studies, US images were used to spatially co-register PA and US image data from days 0, 7 and 14 for each rat. The tibia and fibula, as well as the gastrocnemius, were clearly visible and two-dimensional cross-section slices and their locations were used as fiducial markers in the manual co-registration process. After successful co-registration, manual segmentation was done on PA data to quantify the volume of the region of high-signal corresponding to injection site of AuNP labeled MSCs (as seen in Figure 8). This co-registered segmented data was analyzed to assess the volume of AuNP labeled cells at each time point, for both rats. The total region volume selected for segmentation was $23.1 \times 7.5 \times 4.2 \text{ mm}^3$, lateral $\times$ axial $\times$ elevation, respectively.

Statistical analysis

All test groups consisted of a sample number of 5. Statistical analysis was performed by first performing a Shapiro-Wilk test to evaluate if the data was normally distributed. For data that was normally distributed, a one-way ANOVA followed by student t-tests comparing treatment groups to no treatment were performed using a Bonferroni corrected p value. Results are shown as means $\pm$ standard deviation. For data that was not normally distributed, a Kruskal-Wallis test followed by Mann-Whitney tests comparing treatment groups to no treatment were performed using a Bonferroni correct p value. Results are shown as box plots, where the boxes show the interquartile range and the horizontal lines show the median.

Results

Assessment of MSC viability following syringe injection

The *in vivo* delivery of the stem cells via syringe injection was evaluated to determine if this delivery method was feasible and did not damage MSCs. Cell viability following injection within a PEGylated fibrin gel was evaluated *in vitro* using a LIVE/DEAD stain. Stem cells which were passed through a 23G needle did not undergo cell death, as demonstrated by the large extent of viable cells (Figure S1). Control conditions consisted of cells encapsulated within a PEGylated fibrin gel which had not been passed through a syringe, and a dead control for staining purposes.

Perfusion of ischemic tissue

This study used a rodent hind limb ischemia model in which the femoral artery was ligated and excised (~0.5 cm). Twenty four hours after ligation, animals received either no treatment or intramuscular injection of PEGylated fibrin gel, MSCs, or MSCs + PEGylated fibrin gel into the gastrocnemius muscle of the ischemic hind limb.

Evaluation of immature blood vessel formation was conducted by quantifying lectin positive endothelial cells (Figure 2). In order to account for differences in the muscle architecture between groups, the results were normalized by the number of muscle fibers. Compared to the no treatment group, the MSC + PEGylated fibrin gel group had a significantly higher number of endothelial cells on day 7. On day 14, all treatment groups, with the exception of the serum group, had significantly higher endothelial cells per muscle fiber compared to no treatment.

Quantification of mature blood vessel formation in the treatment groups was performed using smooth muscle actin (SMA) staining (Figure 3). Two quantification methods were used in which the total number of SMA positive vessels (vessels with an area greater than 5 μm^2) and SMA positive area per vessel was evaluated. Quantification of the total number of SMA positive vessels (Figure 3B) demonstrated significantly enhanced blood vessel formation for the PEGylated fibrin gel and MSCs + PEGylated fibrin gel group on day 14. Quantification of the SMA positive area per vessel (Figure 3C) showed no differences between treatment groups on day 7, but significantly enhanced blood vessel formation for the MSCs + PEGylated fibrin gel group on day 14. It should be noted that the serum group had significantly lower SMA positive area per blood vessel compared to the no treatment group. We also quantified the number of SMA positive vessels for the contralateral limbs of no treatment animals. The contralateral limbs had a significantly higher number of SMA positive vessels compared to the ligated limbs for all animals in the no treatment group for both day 7 and day 14 (data not shown). This demonstrates that the ligation procedure lead to a decrease in the number of blood vessels for the ligated limb. Furthermore, since the no treatment group is used as the comparative group in the study, the significant differences we observe in terms of enhanced vasculature for the MSC + PEGylated fibrin gel treatment group is due to the treatment itself.

Reperfusion of the ischemic hind limb was evaluated using speckle imaging and calculating the reperfusion of the ischemic hind limb as a percentage of the contralateral control limb. On day 14 there was a general trend of improved blood flow for all treatment groups excluding the serum group, most notably for the MSC + PEGylated fibrin group, but the differences in blood flow were not significantly different compared to the no treatment group (Figure 4).

Functional recovery of ischemic tissue

In order to evaluate the localization and distribution of MSCs following injection, the cells were fluorescently labeled prior to delivery. Figure S2 shows the distribution of the cells for the MSC and MSC + PEGylated fibrin gel groups at day 7 and day 14. The cells are

distributed throughout the muscle fibers and are present until day 14. However, as evidenced in Figure 5, many of the injected MSCs are not viable at day 7.

Tissue necrosis is typically evaluated histologically by the presence of multi-cellular infiltrates and muscle fibers which are inhomogeneous in size, hypereosinophilic, and devoid of nuclei.[16] Histological analysis of all treatment groups on day 7 and day 14 showed no evidence of inflammation or necrosis, even for the no treatment group (Figure 6).

Functional recovery of the ischemic tissue was evaluated using force production measurements (Figure 7). Functional assessments (quantified as the % of contralateral control) at day 7 showed no significant improvements in specific tension for any of the treatment groups. However, by day 14 there was a significant improvement in specific tension for MSCs + PEGylated fibrin gel ($91.26 \pm 4.06\%$) compared to no treatment ($67.11 \pm 9.40\%$). MSC, PEGylated fibrin gel, and serum treatment groups did not have significant improvements in force production, with specific tension values of $71.74 \pm 9.21\%$, $68.12 \pm 6.23\%$, and $59.03 \pm 8.69\%$, respectively. It should be noted that these force production improvements are dependent on improvements in specific tension (force/cross-sectional area) of the muscle and not just changes in muscle mass (Table 1).

Longitudinal photoacoustic imaging of AuNP labeled MSCs

Combined photoacoustic/ultrasound was performed in order to visualize MSC localization and persistence following intramuscular injection in either PEGylated fibrin gel (MSC + Gel) or 10% FBS cell culture media (MSC) (Figure 8). While the injected volume was equivalent for both treatments, the area of the volume visible in the PA data was much greater for the MSCs with gel treatment ($22.9 \pm 0.8 \text{ mm}^3$) compared to the MSCs only treatment ($6.8 \pm 0.2 \text{ mm}^3$). The most dramatic volume change occurred for the MSCs only treatment at day 7, wherein there was a $25.6 \pm 3.2\%$ reduction in the volume of PA signal as compared to day 0. At the same time point, the MSCs with gel treatment saw only an $8.9 \pm 2.9\%$ reduction in PA signal volume. By day 14 the volume reduction of both the MSCs with gel and MSCs only treatment were roughly the same ($29.6 \pm 2.8\%$ and $33.5 \pm 3.7\%$, respectively). It should be noted that the injection caused a small, but noticeable, increase in the size of the gastrocnemius muscle for both rats. This effect was observable both visually and in US images, but appeared to resolve by day 7 of imaging,

Discussion

Cardiovascular diseases (CVDs) are the number one cause of death globally,[7] and peripheral artery disease and critical limb ischemia in particular affects millions of individuals. Cell-based therapies are widely being investigated as alternative strategies for the currently available revascularization techniques. Many research groups have demonstrated blood vessel formation and increased reperfusion of ischemic areas following delivery of MSCs or other stem cells,[2, 11, 12] either through direct cell differentiation or paracrine effects [7, 10, 11]. Clinical trials involving therapeutic delivery of MSCs to ischemic skeletal muscle have demonstrated improved muscle function and vascular regeneration, [3] although the level of improvement has uniformly been less than hoped.

Biomaterial delivery systems may serve to aid in stem cell therapy by retaining the cells at the delivery site and modulating the local cellular environment.

Previous work by our group demonstrated that PEGylated fibrin promotes the angiogenic protein expression of encapsulated bone marrow derived MSCs without the addition of soluble factors. [14] PEGylation slows fibrinolysis while still maintaining cell viability and upregulating angiogenic and anti-inflammatory gene and protein expression by MSCs. *In vitro* studies by our group has demonstrated that PEGylation leads to a dramatic increase in tubular network formation by MSCs [14, 17, 18] as well as secretion of significantly larger quantities of VEGF and MMP-2 than cell in fibrin gels [19]. These mechanisms could potentially be attributing to the results in the current study, in which animals treated with MSCs + PEGylated fibrin gel exhibited significantly more blood vessel formation and functional muscle recovery.

The current study only investigated PEGylated fibrin gels as a biomaterial system for MSC delivery and therapeutic application. However, based on previous studies conducted in our lab, we believe the results observed in the current study are a function of the hydrogel used. Multiple studies in our group have compared the function of MSCs within PEGylated fibrin gel vs fibrin gel and have found substantial differences [14, 17–21]. These include differences in cell proliferation, gene and protein expression, cytokine secretion, and cell morphology. Specifically, cells were found to form more robust tubular networks, including intercellular vacuole fusion, in PEGylated fibrin gels [19, 21]. These results were also confirmed in *in vivo* studies using a subcutaneous gel plug model [14]. In addition, MSCs in PEGylated fibrin gel secrete significantly larger quantities of VEGF and MMP-2 and have higher expression of vWF and VE-cadherin compared to MSCs in fibrin gel [19]. Furthermore, our group also conducted a study comparing ASC behavior in PEGylated fibrin gels, fibrin gels, and collagen gels [21]. In this study, cells in PEGylated fibrin gels had significantly more extensive network formation compared to fibrin gels and collagen. The cells also expressed decreased levels of SMA in fibrin and PEGylated fibrin compared to collagen on day 7 and slightly upregulated vWF expression. VEGF secretion per cell was significantly higher in PEGylated fibrin compared to fibrin and ELISA-based arrays revealed that ASCs in fibrin-based gels secreted more soluble angiogenic factors than ASCs in collagen, both in quantity and diversity.

In this study, we used a hindlimb ischemia model in order to evaluate the vascular and functional repair of ischemic tissue following stem cell therapy. Specifically, we compared the therapeutic contribution of MSCs delivered within a PEGylated fibrin gel to no treatment. We also evaluated serum (sham treatment), MSCs only, and PEGylated fibrin gel only treatments. The serum treatment group demonstrated that the injection process itself was not stimulating vascular or tissue repair mechanisms, as there were no significant improvements in any of the evaluated measures for the serum group compared to no treatment. Stem cell treatment alone resulted in immature blood vessel formation, with a significantly higher number of endothelial cells in these treatment groups compared to the no treatment group on day 14. MSCs have been shown to secrete factors which are pro-angiogenic and promote wound healing (*e.g.* vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin, platelet-derived growth factor (PDGF), stromal

cell-derived factor (SDF)-1, interleukin-6, matrix metalloproteinase (MMP)-9, transforming growth factor (TGF)- β , macrophage inflammatory protein (MIP), and monocyte chemoattractant protein (MCP)).[7, 9–11] In addition, MSCs demonstrate an enhanced regenerative potential and angiogenic response under hypoxia (<2% oxygen).[22–26] Stem cells have also been shown to secrete immunomodulatory factors, including indoleamine 2,3-dioxygenase (IDO),[27] interferon-gamma (IFN- γ),[27] tumor necrosis factor-alpha (TNF- α),[27, 28] macrophage inflammatory protein (MIP),[9] and interleukins[28, 29] when exposed to hypoxia. As a result, the paracrine action of MSCs can modulate surrounding host cells, [9, 30] thus angiogenesis at day 7 in our study as a result of stem cell delivery is likely due to cytokine secretion.

The low MSC survival rate demonstrated in literature reports following delivery *in vivo* also suggests that delivered cells may play a primarily paracrine role in healing.[31] We also saw low MSC survival within our ischemia model, as demonstrated by TUNEL staining. Furthermore, our previous work using a nanoparticle system to monitor infiltrating macrophages in response to MSCs delivered via PEGylated fibrin following ischemia demonstrated that macrophages infiltrate the injured muscle at the site of delivery. [15] Macrophages are known to have key roles in wound healing and dynamic changes in macrophage populations are essential for efficient tissue repair.[32] Thus, MSCs may indirectly contribute to angiogenesis and skeletal muscle recovery through interactions with macrophages which are localized to the site as a result of the presence of the PEGylated fibrin matrix. In addition, PEGylation of the gel enhances the mechanical properties and slows fibrinolysis, thus enhancing the therapeutic window of MSCs localized within the matrix. [19]

In order for complete revascularization of ischemic tissue to occur, mature blood vessel formation is necessary. We evaluated mature blood vessel formation by staining for the pericyte marker smooth muscle actin. Two different quantification methods were performed in order to compare mature blood vessel formation in the different treatment groups. The SMA positive area per blood vessel was evaluated to compare the average area of blood vessels among groups and to ensure that we were comparing SMA-positive blood vessels and not just myofibroblasts. The other quantification method compared the total number of SMA positive vessels among groups. The PEGylated fibrin gel groups (PEGylated fibrin gel only or MSC + PEGylated fibrin gel) had significantly higher mature blood vessel formation compared to no treatment on day 14 when quantifying the number of SMA positive blood vessels. In addition, quantification of the SMA positive area per blood vessel demonstrated that the MSC + PEGylated fibrin gel group had a significant increase in the area of these vessels on day 14 compared to no treatment. Fibrin has a high affinity for various growth factors and cytokines (including pro-angiogenic factors such as VEGF [33], IGF [34], and TGF- β [35]) and supports angiogenesis and tissue repair.[36] This could account for the enhanced blood vessel formation and maturation towards larger size vessels in the PEGylated fibrin gel treatment group. Fibrin-based matrices are an attractive material for wound healing purposes because fibrin is biocompatible, has high affinity to various biological surfaces, supports angiogenesis and tissue repair, and contains sites for cellular binding.[36] Interestingly, the MSC only group did not exhibit significant vessel formation. These results are in line with the speckle imaging data, in which the PEGylated fibrin gel

groups had improved blood flow to the ischemic limb by day 14, although this difference was not significant.

While revascularization of ischemic tissue is essential, it may not lead to recovery of the tissue. We evaluated the functional recovery of the ischemic tissue following treatment using force production measurements. The only treatment group which resulted in significantly higher functional recovery of the tissue was the MSC + PEGylated fibrin group (around 91%) by day 14. The PEGylated fibrin only group did not show significant improvements in force production, even though these animals exhibited significant blood vessel formation, as demonstrated by lectin and SMA staining, however, SMA vessel size did not increase. Thus, capillary revascularization of ischemic tissue, while necessary for long term recovery, may not translate into early stage functional recovery.

We hypothesize that the MSC + PEGylated fibrin gel group led to significant improvements in force production because the MSCs are contributing to functional recovery and restoration of the ischemic tissue. However, the PEGylated fibrin gel microenvironment appears to modulate the MSCs, as the MSC only treatment group did not have significant improvements in force production at day 14. The gel assists in retaining the MSCs at the delivery site, allowing the cells to contribute to skeletal muscle regeneration through paracrine effects. The US/PA imaging studies of AuNP labeled MSCs demonstrate that MSCs injected within a PEGylated fibrin gel persist in the muscle for at least 14 days, with a greater percentage of cells persisting at later time points in the gel versus without the gel. While this does not measure engraftment or cell survival, we do not have evidence that the cells are engrafting or differentiating towards contractile cells. However, US/PA imaging offers sufficient penetration depth (several centimeters), sub-millimeter spatial resolution, and the capability of visualizing morphological and functional, properties to allow for *in vivo* imaging and visualization of MSCs following injection within our hindlimb ischemia model. Additional mechanisms by which the PEGylated fibrin gel microenvironment may contribute to the observed functional recovery of the tissue may include that the gel could potentially be able to act as a depot to collect cytokines and growth factors, either released endogenously or from the delivered MSCs which may affect the MSCs and surrounding cells. Finally, the interaction of MSCs with infiltrating macrophages that are localized to the PEGylated fibrin may induce changes in macrophage phenotype necessary for skeletal muscle regeneration [37–40].

Interestingly, although the ischemia model investigated here did not appear to result in widespread damage when evaluated histologically (such as cell infiltration and necrosis), functional deficits still occurred. This lack of visual tissue damage may be due to the fact that the rat underused the ligated limb following surgery and also that collateral flow was sufficient to provide blood to the tissue, especially at later time points.[41–43]. Others have seen functional deficits in hind limb ischemia models using femoral artery ligation with no corresponding histological evidence of inflammatory cell infiltration, fibrosis, or edema.[41, 42, 44, 45] The results presented here are in agreement with these other studies, in which consistent, moderate ischemia is associated with less severe inflammation and necrosis compared to acute, severe ischemia.[41, 42, 44, 45]

Conclusions

This study investigated the use of mesenchymal stem cell therapy for ischemic injury. MSCs delivered within a PEGylated fibrin gel contributed to significant improvements in blood vessel formation and arterialization, as well as functional recovery of the ischemic limb, on day 14. While MSCs delivered without PEGylated fibrin exhibited initial signs of vascular repair, and PEGylated fibrin treatment led to enhanced blood vessel formation, neither treatment led to significant functional improvements on day 14. These results suggest that while capillary vasculogenesis is necessary for long term viability, early measures of blood vessel formation may not be predictive of regeneration and functional recovery in the longer term. Furthermore, the results demonstrate that MSCs delivered in combination with PEGylated fibrin gel is a promising therapy for treatment of ischemic diseases. This therapeutic mechanism is attributed to the delivered stem cells, which are known to secrete pro-angiogenic factors and recruit other cell types for wound healing. The gel microenvironment may also play a role by not only altering the function of the stem cells, but also influencing the interaction with endogenous cells types (e.g. macrophages). Furthermore, the gel could act as a depot for released cytokines and growth factors, resulting in a prolonged therapeutic effect. Overall, this study demonstrates that while MSCs and PEGylated fibrin gel show initial therapeutic benefits, long term benefits are only realized when MSCs are delivered in combination with PEGylated fibrin gel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part the National Institutes of Health under grant #R01EB015007 and by a National Science Foundation Graduate Research Fellowship awarded to Laura M. Ricles. The authors would like to acknowledge Noah J. Kopcho for assistance with cryosectioning, Alexandra N. Willauer for assistance with SMA staining, and Elda A. Treviño for assistance with the syringe viability study. The authors would also like to thank Dr. Aaron Baker's laboratory for access to the speckle imaging system.

References

1. Creager MA. Medical management of peripheral arterial disease. *Cardiol Rev.* 2001; 9:238. [PubMed: 11405904]
2. Aranguren XL, McCue JD, Hendrickx B, Zhu XH, Du F, Chen E, Pelacho B, Penuelas I, Abizanda G, Uriz M, Frommer SA, Ross JJ, Schroeder BA, Seaborn MS, Adney JR, Hagenbrock J, Harris NH, Zhang Y, Zhang X, Nelson-Holte MH, Jiang Y, Billiau AD, Chen W, Prosper F, Verfaillie CM, Luttun A. Multipotent adult progenitor cells sustain function of ischemic limbs in mice. *The Journal of clinical investigation.* 2008; 118:505. [PubMed: 18172550]
3. Lawall H, Bramlage P, Amann B. Stem cell and progenitor cell therapy in peripheral artery disease. A critical appraisal. *Thromb Haemost.* 2010; 103:696. [PubMed: 20174766]
4. Taghavi S, Duran JM, George JC. Stem cell therapy for peripheral arterial disease: a review of clinical trials. *Stem Cell Studies.* 2011; 1:105.
5. Epstein SE, Fuchs S, Zhou YF, Baffour R, Kornowski R. Therapeutic interventions for enhancing collateral development by administration of growth factors: basic principles, early results and potential hazards. *Cardiovasc Res.* 2001; 49:532. [PubMed: 11166266]

6. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res.* 2004; 94:678. [PubMed: 14739163]
7. Copland IB. Mesenchymal stromal cells for cardiovascular disease. *J Cardiovasc Dis Res.* 2011; 2:3. [PubMed: 21716750]
8. Appasani, K.; Appasani, RK. Stem cells & regenerative medicine : from molecular embryology to tissue engineering. New York: Humana Press; 2011.
9. Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS one.* 2008; 3:e1886. [PubMed: 18382669]
10. Kinnaird T, Stabile E, Burnett MS, Epstein SE. Bone-marrow-derived cells for enhancing collateral development: mechanisms, animal data, and initial clinical experiences. *Circ Res.* 2004; 95:354. [PubMed: 15321945]
11. Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, Fuchs S, Epstein SE. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation.* 2004; 109:1543. [PubMed: 15023891]
12. Ikenaga S, Hamano K, Nishida M, Kobayashi T, Li TS, Kobayashi S, Matsuzaki M, Zempo N, Esato K. Autologous bone marrow implantation induced angiogenesis and improved deteriorated exercise capacity in a rat ischemic hindlimb model. *The Journal of surgical research.* 2001; 96:277. [PubMed: 11266284]
13. Mosesson MW. Fibrinogen and fibrin structure and functions. *J Thromb Haemost.* 2005; 3:1894. [PubMed: 16102057]
14. Zhang G, Drinnan CT, Geuss LR, Suggs LJ. Vascular differentiation of bone marrow stem cells is directed by a tunable three-dimensional matrix. *Acta Biomater.* 2010; 6:3395. [PubMed: 20302976]
15. Ricles LM, Nam SY, Trevino EA, Emelianov SY, Suggs LJ. A Dual Gold Nanoparticle System for Mesenchymal Stem Cell Tracking. *J Mater Chem B Mater Biol Med.* 2014; 2:8220. [PubMed: 25709814]
16. Limbourg A, Korff T, Napp LC, Schaper W, Drexler H, Limbourg FP. Evaluation of postnatal arteriogenesis and angiogenesis in a mouse model of hind-limb ischemia. *Nat Protoc.* 2009; 4:1737. [PubMed: 19893509]
17. Zhang G, Wang X, Wang Z, Zhang J, Suggs L. A PEGylated fibrin patch for mesenchymal stem cell delivery. *Tissue Eng.* 2006; 12:9. [PubMed: 16499438]
18. Rytlewski JA, Geuss LR, Anyaeji CI, Lewis EW, Suggs LJ. Three-dimensional image quantification as a new morphometry method for tissue engineering. *Tissue Eng Part C Methods.* 2012; 18:507. [PubMed: 22224751]
19. Rytlewski JA, Alejandra Aldon M, Lewis EW, Suggs LJ. Mechanisms of tubulogenesis and endothelial phenotype expression by MSCs. *Microvasc Res.* 2015; 99:26. [PubMed: 25711526]
20. Zhang G, Hu Q, Braunlin EA, Suggs LJ, Zhang J. Enhancing efficacy of stem cell transplantation to the heart with a PEGylated fibrin biomatrix. *Tissue Eng Part A.* 2008; 14:1025. [PubMed: 18476809]
21. Chung E, Rytlewski JA, Merchant AG, Dhada KS, Lewis EW, Suggs LJ. Fibrin-based 3D matrices induce angiogenic behavior of adipose-derived stem cells. *Acta biomaterialia.* 2015; 17:78. [PubMed: 25600400]
22. Muscari C, Giordano E, Bonafe F, Govoni M, Pasini A, Guarnieri C. Priming adult stem cells by hypoxic pretreatments for applications in regenerative medicine. *J Biomed Sci.* 2013; 20:63. [PubMed: 23985033]
23. Grayson WL, Zhao F, Izadpanah R, Bunnell B, Ma T. Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. *J Cell Physiol.* 2006; 207:331. [PubMed: 16331674]
24. Hu X, Yu SP, Fraser JL, Lu Z, Ogle ME, Wang JA, Wei L. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. *J Thorac Cardiovasc Surg.* 2008; 135:799. [PubMed: 18374759]

25. Hoffmann J, Glassford AJ, Doyle TC, Robbins RC, Schrepfer S, Pelletier MP. Angiogenic effects despite limited cell survival of bone marrow-derived mesenchymal stem cells under ischemia. *Thorac Cardiovasc Surg.* 2010; 58:136. [PubMed: 20379963]
26. Yang D, Wang W, Li L, Peng Y, Chen P, Huang H, Guo Y, Xia X, Wang Y, Wang H, Wang WE, Zeng C. The relative contribution of paracrine effect versus direct differentiation on adipose-derived stem cell transplantation mediated cardiac repair. *PloS one.* 2013; 8:e59020. [PubMed: 23527076]
27. Roemeling-van Rhijn M, Mensah FK, Korevaar SS, Leijds MJ, van Osch GJ, Ijzermans JN, Betjes MG, Baan CC, Weimar W, Hoogduijn MJ. Effects of Hypoxia on the Immunomodulatory Properties of Adipose Tissue-Derived Mesenchymal Stem cells. *Front Immunol.* 2013; 4:203. [PubMed: 23882269]
28. Li Z, Wei H, Deng L, Cong X, Chen X. Expression and secretion of interleukin-1beta, tumour necrosis factor-alpha and interleukin-10 by hypoxia- and serum-deprivation-stimulated mesenchymal stem cells. *Febs J.* 2010; 277:3688. [PubMed: 20681988]
29. Chen L, Xu YB, Zhao JL, Zhang ZQ, Yang RH, Xie JL, Liu XS, Qi SH. Conditioned Medium from Hypoxic Bone Marrow-Derived Mesenchymal Stem Cells Enhances Wound Healing in Mice. *PloS one.* 2014; 9
30. Kim J, Hematti P. Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages. *Exp Hematol.* 2009; 37:1445. [PubMed: 19772890]
31. Suga H, Glotzbach J, Sorkin M, Longaker M, Gurtner G. Paracrine mechanism of angiogenesis in adipose-derived stem cell transplantation. *Annals of Plastic Surgery.* 2014; 72:234. [PubMed: 23636112]
32. Ploeger DT, Hosper NA, Schipper M, Koerts JA, de Rond S, Bank RA. Cell plasticity in wound healing: paracrine factors of M1/ M2 polarized macrophages influence the phenotypical state of dermal fibroblasts. *Cell Commun Signal.* 2013; 11:29. [PubMed: 23601247]
33. Sahni A, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. *Blood.* 2000; 96:3772. [PubMed: 11090059]
34. Hammers DW, Sarathy A, Pham CB, Drinnan CT, Farrar RP, Suggs LJ. Controlled release of IGF-I from a biodegradable matrix improves functional recovery of skeletal muscle from ischemia/reperfusion. *Biotechnol Bioeng.* 2012; 109:1051. [PubMed: 22095096]
35. Stowers RS, Drinnan CT, Chung EN, Suggs LJ. Mesenchymal stem cell response to TGF-beta 1 in both 2D and 3D environments. *Biomater Sci-Uk.* 2013; 1:860.
36. Shaikh FM, Callanan A, Kavanagh EG, Burke PE, Grace PA, McGloughlin TM. Fibrin: a natural biodegradable scaffold in vascular tissue engineering. *Cells Tissues Organs.* 2008; 188:333. [PubMed: 18552484]
37. Chazaud B, Brigitte M, Yacoub-Youssef H, Arnold L, Gherardi R, Sonnet C, Lafuste P, Chretien F. Dual and beneficial roles of macrophages during skeletal muscle regeneration. *Exerc Sport Sci Rev.* 2009; 37:18. [PubMed: 19098520]
38. Kharraz Y, Guerra J, Mann CJ, Serrano AL, Munoz-Canoves P. Macrophage Plasticity and the Role of Inflammation in Skeletal Muscle Repair. *Mediat Inflamm.* 2013
39. Novak ML, Koh TJ. Phenotypic transitions of macrophages orchestrate tissue repair. *Am J Pathol.* 2013; 183:1352. [PubMed: 24091222]
40. Tidball JG. Inflammatory processes in muscle injury and repair. *Am J Physiol Regul Integr Comp Physiol.* 2005; 288:R345. [PubMed: 15637171]
41. Brown MD, Kent J, Kelsall CJ, Milkiewicz M, Hudlicka O. Remodeling in the microcirculation of rat skeletal muscle during chronic ischemia. *Microcirculation.* 2003; 10:179. [PubMed: 12700586]
42. Milkiewicz M, Hudlicka O, Shiner R, Egginton S, Brown MD. Vascular endothelial growth factor mRNA and protein do not change in parallel during non-inflammatory skeletal muscle ischaemia in rat. *J Physiol.* 2006; 577:671. [PubMed: 16990404]
43. Peck MA, Crawford RS, Abularrage CJ, Patel VI, Conrad MF, Yoo JH, Watkins MT, Albadawi H. A functional murine model of hindlimb demand ischemia. *Ann Vasc Surg.* 2010; 24:532. [PubMed: 20363101]

44. Tang GL, Chang DS, Sarkar R, Wang R, Messina LM. The effect of gradual or acute arterial occlusion on skeletal muscle blood flow, arteriogenesis, and inflammation in rat hindlimb ischemia. *J Vasc Surg.* 2005; 41:312. [PubMed: 15768015]
45. Yang Z, von Ballmoos MW, Diehm N, Baumgartner I, Kalka C, Di Santo S. Call for a reference model of chronic hind limb ischemia to investigate therapeutic angiogenesis. *Vascul Pharmacol.* 2009; 51:268. [PubMed: 19619670]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. Schematic illustrating the sites of ligation immediately distal to the inferior epigastric artery and proximal to the branch point of the popliteal and saphenous arteries. The femoral artery was ligated and excised (~0.5 cm portion) in order to induce hind limb ischemia.

Figure 2.

(A) Lectin staining of ischemic muscles with various treatments, and contralateral control, at day 7 and 14. (B) Quantification of lectin positive blood vessels normalized by the number of muscle fibers. MSCs delivered within a PEGylated fibrin gel exhibited significantly more lectin positive blood vessels at both day 7 and day 14, while all treatment groups had significantly more lectin positive blood vessels at day 14 compared to no treatment. (* = $p < 0.0125$ compared to no treatment)

Figure 3.

(A) Smooth muscle actin (SMA) staining of ischemic muscles with various treatments, and contralateral control, at day 7 and 14. Quantification of (B) the number of SMA positive vessels (average value $\pm$ standard deviation) and (C) SMA positive area per vessel. MSCs delivered within a PEGylated fibrin gel had significantly more SMA positive vessels on Day 14 and corresponding SMA positive area per blood vessel on Day 14 compared to no treatment. The PEGylated fibrin gel group had significantly more SMA positive blood vessels on Day 14, indicating the PEGylated fibrin gel has some angiogenic properties. The

serum group had significantly lower SMA positive area per vessel on Day 7 and 14 compared to no treatment. (* = $p < 0.0125$ compared to no treatment)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Evaluation of blood flow to ischemic hind limbs using speckle imaging (average value $\pm$ standard deviation). There were no significant differences among groups.

Figure 5. Evaluation of cell apoptosis following *in vivo* delivery within PEGylated fibrin gel on day 7. MSCs were pre-labeled with CM-DiI (red) and localized within muscle fibers following isolation of the muscle on day 7. TUNEL staining (green) indicated co-localization of apoptotic cells with CM-DiI labeled cells, suggesting that many of the MSCs injected into the ischemic muscle were undergoing apoptosis. Scale bar = 50 μm.

Figure 6. Hematoxylin and eosin staining of ischemic muscles with various treatments, and contralateral control, at day 7 and 14. None of the groups exhibited signs of substantial inflammation or tissue necrosis. Magnified images are shown as insets. Scale bar = 100 μm .

Figure 7. Maximum force production measurements of the lateral gastrocnemius 7 and 14 days following treatment. Black lines indicate mean values for each treatment group (average value $\pm$ standard deviation). MSCs delivered within a PEGylated fibrin gel had significantly improved muscle function recovery ($91.26 \pm 4.06\%$) by day 14 compared to no treatment. (* = $p < 0.0125$ compared to no treatment)

Figure 8.

Combined photoacoustic/ultrasound imaging ($\lambda = 740$ nm) of gold nanoparticle labeled MSCs injected alone or in combination with PEGylated fibrin gel into the lateral gastrocnemius of the ischemic hindlimb. The volume of the photoacoustic signal region decreased by $25.6 \pm 3.2\%$ for the MSC only treatment group on day 7 compared to day 0, whereas the MSC with gel treatment group only saw a signal reduction of $8.9 \pm 2.9\%$. By

day 14 the volume reduction of both the MSCs with gel and MSCs only treatment were roughly the same (29.6 ± 2.8 % and 33.5 ± 3.7 %, respectively).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Muscle parameters, including muscle mass and cross-sectional area, 7 and 14 days following treatment (average value $\pm$ standard deviation).

	Muscle mass (mg)		CSA (cm ²)	
	Day 7	Day 14	Day 7	Day 14
No treatment	869.60 $\pm$ 45.24	855.75 $\pm$ 68.12	0.73 $\pm$ 0.02	0.72 $\pm$ 0.04
MSC	866.40 $\pm$ 47.73	842 $\pm$ 18.76	0.67 $\pm$ 0.27	0.70 $\pm$ 0.04
Gel	862.60 $\pm$ 82.17	964.60 $\pm$ 29.23	0.70 $\pm$ 0.07	0.80 $\pm$ 0.03
MSC + Gel	824 $\pm$ 23.42	770.60 $\pm$ 75.05	0.68 $\pm$ 0.04	0.65 $\pm$ 0.04
Serum	928.25 $\pm$ 61.37	986 $\pm$ 93.26	0.73 $\pm$ 0.03	0.79 $\pm$ 0.06