Human Placenta-Derived Adherent Cells: Angiogenic Properties and Efficacy in Hindlimb Ischemia

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Abstract

Background: Human placenta-derived adherent cells (PDAC® cells) are a culture expanded, undifferentiated mesenchymal-like population from full-term placental tissue and were previously shown to possess anti-inflammatory and immunomodulatory properties. PDAC® cells (formulated as PDA-002) are in clinical trials for Peripheral Artery Disease with Diabetic Foot Ulcer. In the current study, we examined their angiogenic and tissue-reparative properties.

Methods: The effects of PDAC® cells on survival, migration and tube formation of human umbilical vein endothelial cells (HUVEC) were tested using conditioned media and non-contact co-culture. Angiogenic effects were assessed in the chick chorioallantoic membrane (CAM) assay. Hindlimb ischemia (HLI) was induced in mice and rats by femoral artery transection, and blood flow and blood vessel density were monitored in vivo by Doppler and angiography in the ischemic and control limb. Tissue damage and regeneration in HLI were examined in histological sections of quadriceps muscle stained with hematoxylin and eosin, and newly synthesized blood vessels were detected by indoxyl-tetrazolium staining for alkaline phosphatase.

Results: PDAC® cells enhanced the survival of serum-starved HUVEC and stimulated HUVEC migration and tube formation. In the CAM assay, PDAC® cells stimulated blood vessel formation, with efficacy indistinguishable from the fibroblast growth factor control. In HLI, intramuscular administration of PDAC® cells resulted in improved blood flow and vascular density, and in quadriceps muscle, tissue regeneration and increased numbers of blood vessels were observed.

Conclusion: PDAC® cells exhibited various activities consistent with angiogenesis and tissue repair, supporting the continued investigation of this cell therapy as a treatment for vascular disease-related indications.

Keywords: placenta, mesenchymal stromal cells, angiogenesis, ischemia, cell therapy Running title: Angiogenic Properties of Placenta-Derived Cells

Abbreviations:

1) PDAC®: placenta-derived adherent cells
2) CAM: chorioallantoic membrane
3) HGF: Hepatocyte Growth Factor
4) MSC: mesenchymal stromal cells
5) DMEM: Dulbecco's Modified Eagle Medium
6) HUVEC: Human umbilical vein endothelial cells
7) EGM-2: Endothelial Cell Growth Medium-2
8) EBM: Endothelial Cell Basal Medium
9) SF-DMEM: serum-free DMEM
10) HPC: Human Placental Collagen
11) FGF-2: Fibroblast Growth Factor-2
12) G-CSF: Granulocyte- Colony Stimulating Factor
13) IL-8: Interleukin-8
14) PDGF-BB: Platelet-Derived Growth Factor BB
15) VEGF: Vascular Endothelial Growth Factor
16) IL-6: Interleukin-6
17) MCP-1:Monocyte Chemoattractant protein-1
18) siRNA: small interfering RNA
19) HLI: hindlimb ischemia

Introduction

Angiogenesis is a complex process that facilitates blood vessel formation and tissue perfusion. It is associated with a balance of stimulatory and inhibitory steps including secretion of various soluble factors, degradation of extracellular matrix, proliferation and migration of endothelial cells, and differentiation of endothelial cells into tube-like vessels (). The term “therapeutic angiogenesis” refers to situations where angiogenic stimulation is a clinically desirable outcome; e.g., tissue repair or treatment of local hypovascularity (). To date, no single small-molecule or biologic-based approach for therapeutic angiogenesis has proven consistently efficacious, and a number of compounds that showed promise in preclinical and early clinical trials failed to demonstrate efficacy in larger randomized trials (). These failures could be due to the fact that the processes of angiogenesis and tissue repair are not regulated via a single molecule or biological pathway, but rather by an intricate, temporally coordinated network of biological processes.

It has been suggested that cell therapy using mesenchymal stromal cells (MSCs) or mesenchymal-like cells available from a wide variety of sources such as bone marrow, adipose tissue, and placenta, can promote angiogenesis and tissue regeneration via a plethora of biological activities, offering a potentially transformational means to treat diseases that have significant unmet clinical needs ().. These cell therapies have been shown to modulate pathological immune responses and inflammatory processes (), as well as the secretion of specific trophic factors that stimulate resident endothelial cells and/or progenitors to initiate angiogenic or vasculogenic processes in injured tissue ().

The human placenta from normal, full-term pregnancies is readily available in large supply and is a particularly attractive source for MSC-like cells. Placenta-derived adherent cells (PDAC® cells) are a culture expanded, undifferentiated mesenchymal-like population derived from full-term placental tissue (Liu 2014). The cells have the genotype of the newborn and display features characteristic of MSCs, but with a unique phenotype associated with their placental origin. The cells are anchorage-dependent in culture and adhere to the plastic surface during expansion culture in vitro (). PDAC® cell-mediated activities have been studied in both in vitro and in animal models and display immunomodulatory, anti-inflammatory, pro-regenerative and neuroprotective properties (Liu 2014, Shehadah 2014, He 2013) suggesting that the cells may have potential utility across a variety of disease

indications. PDA-001 (cenplacel-L), an i.v. formation of PDAC® cells, is in clinical trials for various autoimmune and inflammatory diseases, including Crohn's Disease (Mayer 2013). PDA-002, an intramuscular formulation of PDAC® cells, is in clinical development for the treatment of diabetic foot ulcer in the setting of peripheral arterial disease (http://clinicaltrials.gov/show/NCT01859117).

In this study, we sought to determine whether in addition to previously described immunomodulatory capabilities, PDAC® cells also display angiogenic properties in various in vitro and ex vivo assays and in animal models relevant to angiogenesis. We show that PDAC® cells secrete a variety of proangiogenic factors (most prominently Hepatocyte Growth Factor [HGF], induce blood vessel formation, support blood vessel growth and maintenance, and, most importantly, induce muscular repair in rodent hindlimb ischemia models, demonstrating that these cells possess significant proangiogenic and reparative functions. These results support the continued investigation of PDAC® cells as cell therapy for vascular disease-related indications.

Materials and Methods

Preparation of PDAC®cells and human umbilical vein endothelial cells (HUVEC)

PDAC® cells were prepared as described by mechanical and enzymatic digestion of human placental tissue of newborn origin obtained from a normal, full term birth (Liu 2014). PDAC® cells were expanded and maintained until passage 6 (p6) in PDAC® medium (Liu 2014). Unless otherwise indicated, PDAC® medium contains 2% fetal bovine serum (Invitrogen, Carlsbad, CA). Cryopreserved PDAC® cells (frozen at p6 at 7.5 x 10^6 or 20 x 10^6 cells/mL in 5% dimethyl sulfoxide, 10% human serum albumin, and 5.5% dextran in 0.9% NaCl) were quickly thawed in a 37°C water bath. Viability and cell counts were assessed by trypan blue exclusion. The cells were then diluted to appropriate concentrations and used within 4 hours of thaw.

HUVEC were obtained from Lonza (Walkersville, MD) and were culture-expanded according to the manufacturer's instructions and used in experiments at passage 3 (p3).

Preparation of cell conditioned media (CM)

PDAC® cells were seeded onto tissue culture-treated polystyrene (TCPS) 24-well plates (Corning, Lowell, MA) at 4000 cells/cm^2 for overnight establishment in PDAC® medium prior to incubation in serum-free (SF) PDAC® medium for 48 hours (37ºC, 5% CO_2, 21% O_2, 90% relative humidity). Upon completion of incubation, CM was collected and frozen at -80ºC prior to subsequent analyses as described below.

HUVEC cell counts after serum starvation

HUVEC were subjected to serum starvation in the presence and absence of PDAC® cells, using indirect co-culture systems. HUVEC were seeded at 2x10^4 cells in each well of a 24-well TCPS pre-coated with fibronectin (Sigma, St. Louis, MO) as per the manufacturer's instructions and were incubated overnight in complete Endothelial Cell Growth Medium-2 (EGM-2) (Lonza, Walkersville, MD) to facilitate cell attachment and proliferation. Following overnight incubation, the cells were serum-starved in Endothelial Cell Basal Medium (EBM) (Lonza, Walkersville, MD) for 6 hours.

At the time of HUVEC seeding, 0-5 x 10^4 PDAC® cells were independently seeded into 8 μm-pore transwell culture inserts (Corning, Lowell, MA) in a separate multiwell plate in PDAC® medium and incubated for 2 hours to allow

attachment. Subsequently, the PDAC® medium was replaced with SF-Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) for 24 hours in order to prepare CM containing factors secreted by PDAC® cells. The following day, medium was removed from the HUVEC cultures and replaced with PDAC® CM. Additionally, the PDAC® cells transwell inserts were placed into the HUVEC multiwell plates to produce an indirect co-culture between PDAC® cells, and HUVEC that were overlaid with PDAC® CM. After 24 hours, the transwell co-cultures and media were removed, and the effect of PDAC® cells and CM on HUVEC cell count was determined.

HUVEC cell counts were determined using the Cell Titer Glo assay (Promega, Madison, WI). This assay correlates cellular ATP concentrations with cell number and metabolic activity via a luminescent ATP reporter reagent. Luminescence was quantified using a Bio-Tek® Synergy™ HT Multi-Detection Microplate reader (Winooski, VT). Cell numbers were calculated from a standard curve generated on the day of cell seeding. The lower limit of detection and dynamic range of this assay were ~5,000 and 5,000-90,000 HUVEC, respectively. Unconditioned SF-DMEM was used as a control to evaluate the effect of PDAC® cells, and EGM-2 was used as an assay control.

Transwell cell migration assay

The CytoSelect™ 24-Well Cell Migration Assay Kit, 8 μm (Cell Biolabs San Diego, CA) was used to measure HUVEC migration. Briefly, 5 x 10^4 PDAC® cells were seeded into a 24-well plate and allowed to attach overnight in PDAC® medium. Following overnight incubation, the medium was changed to SF-DMEM to initiate conditioning for 24 hours. On the day of migration experiments, HUVEC were resuspended in basal EBM to a concentration of $5x10^5$ cells/mL, and 50,000 cells were pipetted on top on transwell inserts that were placed in wells containing either the PDAC® cells and their CM, SF-DMEM, or EGM-2. Following a 4-6 hour HUVEC migration time period at 37°C, the transwells were removed, and non-migrated HUVEC were removed by swabbing the inner component of the transwell insert. Migrated cells were stained for 10 minutes in 600 μL of Cell Stain Solution. After staining, transwells were rinsed in dH_2O and air-dried. Dried transwells were placed in 200 μL Extraction Solution to solubilize the dye. A 100 μL sample was transferred to 96-well plates, and absorbance of the dye (560 nm) was measured using a Bio-Tek® Synergy™ HT Multi-Detection Microplate reader (Winooski, VT). Relative absorbances of HUVEC transwells were compared to SF-DMEM (negative control) and EGM-2 (positive control).

Tube formation assay

HUVEC were cultured to p3 in EGM-2 for 3 days and harvested at 70-80% confluency. HUVEC were then washed once with DMEM and resuspended in the same medium at the desired concentrations. The HUVEC suspension was diluted to 4,000 cells/µL with Human Placental Collagen (HPC, 1.5 mg/ml in DMEM) and pipetted into 96-well plates at 3µL per well (n=5 per condition). The HUVEC/HPC drops were incubated for 75-90 min (37ºC , 5% CO_2, and 90% relative humidity) to facilitate collagen solidification, followed by the addition of 200 µL of test medium (PDAC®-CM or control medium) to each well, and incubation was continued for 24 hours. After incubation, the HUVEC drops were stained using a Diff-Quik Cell Staining Kit (Dade Behring, Inc., Newark DE) as recommended by the manufacturer. Images of each well were acquired using the Zeiss SteReo Discovery V8 microscope and analyzed for average network area.

BioPlex Pro™ multiplex assay

The concentrations of angiogenic factors basic Fibroblast Growth Factor-2 (FGF-2), Granulocyte Colony-Stimulating Factor (G-CSF), Interleukin-8 (IL-8), Platelet-Derived Growth Factor BB (PDGF-BB), Vascular Endothelial Growth Factor (VEGF), Interleukin-6 (IL-6), and Monocyte Chemotactic Protein-1 (MCP-1), were analyzed in PDAC® cell-CM using Bio-Plex Pro™ multiplex bead array systems (BioRad Laboratories, Hercules, CA) according to manufacturer's instructions, followed by quantitation using a Luminex™ dual-laser, flow-based microplate analysis system (Luminex 100, Starstation v2.3, Applied Cytometry Systems, Sheffield, UK).

Hepatocyte Growth Factor (HGF) ELISA

Secretion of HGF into culture media was determined using a solid phase sandwich ELISA Immunoassay system (Quantikine Human HGF Immunoassay, R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. HGF concentrations were determined by absorbance of the reporter chromagen (tetramethylbenzidine) at 450 nM using an HGF standard curve (40-8000 pg/mL range).

Reduction of HGF transcripts in PDAC® cells using siRNA

To reduce the amount of HGF transcript in PDAC® cells by transient transfection of small interfering RNA (siRNA), pools of siRNAs (4 per target gene) for human HGF, a non-targeting negative control, and Dharmafect1 transfection reagent were obtained from Dharmacon Inc. (Lafayette, CO) and used according to the manufacturer's instructions. PDAC® cells were seeded into 6-well plates at a density of 10^4 cells/cm^2 (~50% confluence), and

incubated overnight (37ºC, 5% CO_2, and 90% relative humidity). The cells were transfected with final pooled siRNA concentrations of 100 nM and final Dharmafect1 concentrations of 0.2% v/v. Cells were used for analyses 24 hours post-transfection.

Quantitation of gene transcripts

Specific gene transcripts were quantitated using standard quantitative Real Time Polymerase Chain Reaction (qRT-PCR) techniques. Total RNA was extracted from trypsinized cells using RNeasy Mini kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized from 2 µg of total RNA using the Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Each qRT-PCR reaction was performed in triplicate and consisted of 1x Taqman Fast Universal PCR mix, 1x Taqman primers and probes, and 100 ng of cDNA in a 20 µL reaction in a 96 well plate. Acceptable coefficients of variation (CV's) were 1-3%. The PCR thermocycle parameters were 95ºC for 20 s, 40 cycles of 95ºC for 1 s, and 60ºC for 20 s, using an Applied Biosystems 7500 Fast Real Time System. The Taqman primers and probes for human HGF were obtained from pre-validated inventories (Applied Biosystems, Foster City, CA). The expression level of housekeeping gene human β-actin was used to normalize each sample.

Chicken embryo chorioallantoic membrane (CAM) assay

CAM assays in fertilized chicken eggs were used to assess neovascularization and were performed by SRI International (Menlo Park, CA) as described (Ribatti et al, 1996). The test articles applied on Day 6 were: thawed PDAC® cells (7.7×10^5 viable cells) in 40 µL of DMEM/Matrigel vehicle mixture (1:1), positive control (100 ng/mL bFGF in DMEM/Matrigel mixture, Matrigel vehicle control, and DMEM control. Embryos were returned to the incubator after dosing, and on Day 8, blood vessel density was determined using an image capturing system at a magnification of 100×. The blood vessel density was measured using an angiogenesis scoring system (5 = highest density; 0 = no angiogenesis). The percent of vascular growth at each dosing site was calculated using the score recorded for that site divided by the mean score of the control samples for each individual experiment. The percent of vascular growth for each test article was calculated by pooling all results obtained for that dose from 8-10 embryos.

Animal care

All animal work was performed at Pharmaseed Ltd. (Ness Ziona, Israel). Healthy adult Balb/c mice (approximately 25 g) or Sprague Dawley rats (approximately 200 g) were used to assess the angiogenic and reparative activity of PDAC® cells in hindlimb ischemia (HLI) models. Animal handling was according to the National Institute of Health (NIH) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals were fed ad libitum with a commercial rodent diet and had free access to autoclaved and acidified drinking water (pH 2.5 - 3.0). Animals were housed under standard laboratory conditions, air conditioned and filtered (HEPA F6/6) with adequate fresh air supply (minimum 15 air changes/hour) at 20 – 24^{o}C, relative humidity of 30 – 70%, and 12 hours light,12 hours dark cycle.

Unilateral HLI induction and administration of PDAC® cells

The surgical procedure followed the Prox-A protocol described by Goto et al. (2006). Under anesthesia, the animal was placed with ventral side up. An incision (mice, 0.5-1.0 cm; rats, 1.0 – 2.0 cm) was made in the skin in the inguinal area. The femoral artery was ligated twice with 6-0 (mice) or 4-0 (rats) silk thread and transected between the ligatures. The wound was closed with 4-0 (mice) or 3-0 (rats) silk thread, and the animal was allowed to recover.

At 24 hours (mice and rats) post surgery, each animal was treated with PDAC® cells, rhVEGF 165 (3.3 µg/animal, Sigma-Aldrich, USA) or vehicle (cell freezing media) via intramuscular injection proximal and distal to the surgical wound. The mice were injected with 25 µL in each of 2 sites (total 50 µL), and the rats were injected with 50 µL in each of 4 sites (total 200 µL). Animals received PDAC® cell doses of approximately 4 x 10^4, 4 x 10^5, or 4 x 10^6 cells/Kg.

Laser Doppler analysis

Blood flow in the surgical and control (non-surgical) hindlimb was measured with a non-contact laser Doppler before surgery and on days 1, 7 15, 21, 28, and 35 for both mice and rats. Additionally, mice were measured at 42 and 49 days. Blood flow in the non-ischemic control limb is defined as 100%; measurements are expressed as % flow in the ischemic limb compared to the control limb.

Angiography of rat hind limbs

On day 35 post surgery, half of the animals in each group were anesthetized with ketamine/Xylazine. Barium sulfate solution (2.5 ml) was infused into the infrarenal aorta after ligation of the proximal aorta and inferior vena cava, and the number of contrast-filled blood vessels was determined by X-Ray roentgen assays. Quantitation of the number of intersections between contrast-filled vessels and gridlines of the angiographs is presented as an angioscore defined as a number of blocks on a grid filled by the radio-opaque dye in a region of interest which was performed by a blinded observer.

Histology of hindlimb muscle

After sacrifice, the quadriceps muscles of the ischemic and control limbs were removed, weighed, fixed in Hepes-glutamic acid buffer mediated Organic solvent Protection Effect (HOPE) fixative (DCS Innovative, Hamburg, Germany), and embedded in paraffin. The paraffin blocks were cooled to 20°C for 30 min and sectioned using a microtome at 5 μm thickness. Muscle anatomy was visualized by staining two serial sections with hematoxylin and eosin and evaluated microscopically for morphological changes.

Quantitation of blood vessel density in muscle sections

The indoxyl-tetrazolium method for alkaline phosphatase was used to detect capillary endothelial cells in newly synthesized vessels. Staining was performed using BCIP/NBT kit from Vector Labs (Burlingame, CA, cat # SK-5400), and nuclei were counterstained with methyl green (Vector Labs, Cat# H-3402) according to manufacturer's protocol. Muscle capillary density was evaluated in a blinded fashion in sections from three representative animals by counting positively stained capillaries within three randomly chosen fields per section at 20x magnification.

Results

PDAC® cells enhance the survival of endothelial cells

The effect of PDAC® cells on the survival of serum-starved HUVEC was examined in a non-contact co-culture system. Following serum starvation and 24 hrs of indirect co-culture of the two cell types, only 35% of the cell count of HUVEC remained in the absence of PDAC® cells (Figure 1a, SF-DMEM). In contrast, indirect co-culture with PDAC® cells resulted in 72% HUVEC survival. Although we refer to the observed increase in the HUVEC count in the presence of PDAC® cells compared to control as "enhanced survival (reduced cell death)," we cannot exclude the possibility that enhanced HUVEC proliferation may be a component.

Dose-response studies were performed in order to determine the ratio of PDAC® cells to HUVEC required to promote HUVEC survival (Fig. 1b). A range of PDAC® cells (0 – 40,000) was used, while the input HUVEC was kept constant at ~50,000 cells (PDAC®:HUVEC of 0.1:1 to 0.8:1). When cultured in DMEM alone, the HUVEC count decreased by ~60%, to 20,000 cells. As few as 5000 PDAC® cells had a statistically significant effect on the HUVEC count ($p<0.01$), with a plateau observed at approximately 20,000 PDAC®cells, resulting in 2-fold higher final HUVEC count compared to the DMEM control ($p<0.01$). Based on the starting HUVEC count, PDAC® cells exerted a beneficial effect on HUVEC survival at as little as a 0.1:1, with a plateau observed at ~ 0.4:1 PDAC®:HUVEC. The lack of direct contact between PDAC® cells and HUVEC in these experiments suggests that the effect is mediated via factors elaborated by PDAC® cells.

PDAC® cells stimulate endothelial cell migration

The ability of PDAC®cells to stimulate HUVEC migration was examined in a transwell system (Fig 2a). The number of HUVEC (represented by absorbance) that migrated in response to PDAC® cells and CM was ~70% higher compared to the SF-DMEM control, a statistically significant difference ($p<0.01$). The stimulation of HUVEC migration by PDAC® cells and CM was statistically indistinguishable from stimulation by the positive control, EGM-2.

PDAC® cell- CM stimulates endothelial cell tube formation

The ability of CM from PDAC® cells to promote HUVEC tube formation was examined by the application of PDAC®-CM to HUVEC cultured within a three-dimensional environment of collagen. After 24 hours of incubation, tube formation (as measured by average network area) was ~70% greater for CM than for serum-free media ($p<0.01$) (Figure 2b).

Trophic factor protein secretion of PDAC® cells

Since direct PDAC® cell contact with HUVEC was not required to stimulate HUVEC survival, migration, or tube formation (Figs 1 and 2), it was of interest to examine protein secretion by PDAC® cells. Assessment of a set of 8 proteins (Fig. 3) revealed that PDAC® cells secrete numerous mitogens and cytokines relevant to angiogenesis. All 8 factors were detected at levels above the assay lower limit (~10 pg/mL - >2000 pg/mL). Factors FGF-2, G-CSF, IL-8, and PDGF-BB were secreted in low, but detectable levels (<100 pg/mL); VEGF and IL-6 were secreted at moderate levels (140 and 480 pg/mL, respectively). MCP-1 and HGF were secreted at substantially higher levels (~1200 and 1600 pg/mL, respectively). Due to its abundance and known association with a variety of physiological functions (REF), further study of the effect of PDAC® cell-secreted HGF was conducted.

HGF may contribute to PDAC® cell -mediated HUVEC survival

To determine whether HGF was required for PDAC® cell-mediated survival of serum-starved HUVEC in culture as was observed in Fig. 1, HGF expression was inhibited by use of an siRNA that targets HGF transiently transfected into PDAC® cells. siRNA transfection resulted in specific and significant reduction in HGF secretion. RT-PCR analysis (4 independent experiments) consistently demonstrated >90% reduction in HGF transcripts, and HGF protein secretion in the CM of transfected PDAC® cells was undetectable when analyzed with an HGF ELISA (data not shown). PDAC® cells in which HGF expression had been reduced by siRNA displayed a slightly diminished (but statistically significant, $p<0.01$) effect on HUVEC survival (~20% less survival compared to untransfected PDAC® cells, and PDAC® cells transfected with a non-HGF siRNA control, data not shown). These results suggest that HGF contributes in part to PDAC® cell-mediated enhancement of serum-starved HUVEC survival in vitro. As with Fig. 1 data, we cannot exclude a component of PDAC® cell-enhanced HUVEC proliferation in this experiment.

Ex vivo proangiogenic effects of PDAC® cells

To investigate the effect of PDAC® cells on vascularization in an *ex vivo* model, PDAC® cells were cultured on exposed chorioallantoic membranes (CAM) of chick embryos, and the change in blood vessel density was assessed after 8 days of incubation. As shown in Fig. 4, PDAC® cells caused a substantial increase (~5- to 10-fold) in blood vessel density compared to the medium and vehicle controls and was indistinguishable from the positive control FGF-2, a known proangiogenic factor.

PDAC® cell administration increases blood flow in rodent models of hindlimb ischemia (HLI)

The proangiogenic effects of PDAC® cells, and its effects on HUVEC migration, and on survival and/or proliferation of serum-starved cells, led us to test the effects of PDAC® cells in rodent models of HLI. After induction of HLI, blood flow was measured with and without intramuscular administration of PDAC® cells. Throughout the mouse study (Fig. 5a), an increase in blood flow was observed in all animal groups treated with PDAC® cells compared to the vehicle-treated control. The improvement in blood flow was visible from day 28 and continued to increase through day 49 post surgery, similar to the positive control, VEGF. The increase in blood flow in all PDAC® cell treatment groups compared to the vehicle control was statistically significant (from day 28 through day 49, $p<0.01$).

In a rat study, an increase in blood flow was observed in all animal groups treated with PDAC® cells, compared to the vehicle-treated control (Fig. 5b). This improvement was observed on day 35 after HLI initiation in all PDAC® cell- treated groups and was statistically significant compared to the vehicle control ($p< 0.001$ for day 35 at all PDAC® cell doses). To determine whether PDAC® cell viability is required for the observed increase in blood flow, non-viable cells were prepared by repeated freeze-thaw cycles prior to administration in the rat HLI model; non-viable PDAC® cells failed to improve blood flow.

Thus, in both mouse and rat models of HLI, intramuscular injection of PDAC® cells was effective in restoring blood flow to the ischemic limb.

Angiography assessment of rat hind limbs

To further investigate the restoration of blood flow observed in the rat HLI model , blood vessel density was examined by angiography of ischemic limb from rats with and without PDAC® cell treatment, 35 days after induction of ischemia (Fig. 6). By visual inspection, higher blood vessel density was observed upon PDAC® cell treatment (panel B) compared to the vehicle control (panel A) and is quantified in panel C. PDAC® cell treatment resulted in a 2-fold increase in the angioscore compared to the vehicle control ($p < 0.01$ or 0.05 for day 35 at both PDAC® cell doses).

Histology of ischemic limb muscle

To visualize muscle fibers, hematoxylin and eosin stained sections of the mouse quadriceps muscle from ischemic and healthy animal hind limbs were examined (Fig. 7). In cross section, skeletal muscle fibers in the non-ischemic limb had a polygonal appearance with relatively narrow borders of connective tissue (panel a). In ischemic limb (panel b), the myofibers appeared smaller and irregular, with correspondingly larger borders of surrounding connective tissue and the muscle tissue frequently showed inflammatory infiltrates adjacent to blood vessels. Treatment with PDAC® cells (panel d) led to the reduction of inflammatory infiltrates and a marked improvement in the structure of muscle fibers, with an appearance similar to uninjured muscle in the non-ischemic limb (panel a), but with myofibers containing multiple, centrally located nuclei, indicative of tissue regeneration. PDAC® cells had an effect similar to the VEGF control (compare panels c and d); however, PDAC® cell treatment resulted in an apparent further reduction of interspersing connective tissue. The effects of PDAC® cell treatment on rat muscle histology were similar (data not shown).

Murine blood vessel density measurement

Vascular structures in PDAC® cell-treated mice were visualized by alkaline phosphatase staining (Fig. 8 a, b) and quantitated, using VEGF as the positive control (Fig.8 c, d). PDAC® cell treatment (4×10^5/Kg or 4×10^5/Kg) resulted in markedly higher numbers of all sizes of blood vessels (capillaries 50 - 100 μm^2 and 300-700 μm^2; arterioles 300-700 μm^2; and larger blood vessels >700μm^2) in comparison to vehicle controls. VEGF was similar to PDAC® cell treatment in vessel density in the size ranges ≤700 μm^2), but for larger vessels (>700μm^2), PDAC® cell treatment resulted in ~3-fold higher density compared to VEGF. For smaller vessels (≤300 μm^2), VEGF and

PDAC® cell treatment each exceeded the vessel density of the healthy control limb. Thus, histology of muscle taken from the ischemic limb of mice treated with PDAC® cells demonstrated not only muscle regeneration at the level of muscle fibers, but also an increase in the number of blood vessels of all sizes examined, relative to vehicle-treated mice.

Discussion

Human PDAC® cells are a novel MSC-like population previously shown to possess potent immunomodulatory, anti-inflammatory, neuroprotective, and pro-regenerative properties (Liu 2014, Shehadah 2014, He 2013, Chen 2012, Li 2011). In the current study, we explored the angiogenic properties of PDAC® cells. In vitro, PDAC® cells exhibited various activities consistent with angiogenesis including stimulation of endothelial cell migration and tube formation, enhancement of endothelial cell survival and/or proliferation under conditions of serum starvation. PDAC® cells also promoted vascularization in the CAM assay. Importantly, in clinically-relevant disease models of HLI in both rats and mice in vivo, intramuscular injection of PDAC® cells resulted in increased blood flow, increased blood vessel density, and repair of damaged tissue. These studies support continued investigation of PDAC® cells as an angiogenic therapeutic in clinical studies. PDAC cells (formulated as PDA-002 for local injection) are currently in clinical trials for Peripheral Artery Disease with Diabetic Foot Ulcer.

It is becoming evident that treatment of certain vascular diseases with a single factor may have limited efficacy (REF) and may not be sufficient to ameliorate complex pathologies with parallel excessive inflammation and/or tissue degeneration. To date, no single small-molecule or biologic-based approach for therapeutic angiogenesis has proven consistently efficacious, and a number of therapeutic modalities that showed promise in preclinical and early clinical trials failed to demonstrate efficacy in larger randomized trials (REF). Kahn et al. reviewed the results of phase I/II clinical studies that investigated gene therapy approaches to induce therapeutic angiogenesis via sustained expression of select growth factors, such as VEGF and HGF in peripheral artery disease or myocardial ischemia. These studies consistently reported that gene therapy approaches appeared safe, but results were mixed with regard to efficacy and statistical significance (28). Furthermore, a large phase III clinical trial utilizing FGF gene therapy for critical limb ischemia failed to demonstrate any improvement over the placebo group and also failed to meet the study's secondary outcomes (29). This lack of efficacy may be due to the complexity of the processes of angiogenesis and tissue repair, which are likely not regulated via one molecule or biological pathway, but rather by an intricate network of biological activities.

In contrast to single agents, MSCs and mesenchymal-like cells have been referred to as an "injury drugstore" (Caplan 2011), possessing immunomodulatory, anti-inflammatory, pro-regenerative and angiogenic activities and acting in a paracrine fashion to provide trophic support, often without clear evidence of engraftment (Murphy 2013,

etc. etc.). Mesenchymal-like cells from a variety of sources can promote angiogenesis and tissue regeneration via a wide variety of biological activities, offering a potentially transformational means of treating diseases that have significant unmet clinical needs (2, Liew, Bronckaers, DiMarino). Caplan and Correa (2011) posit that MSCs are able to "establish a regenerative microenvironment by secreting bioactive molecules and regulating the local immune response." . Our experiments demonstrate PDAC® cell multifunctionality consistent with these observations. .

Our experiments using conditioned medium from PDAC® cells, and/or non-contact co-culture of HUVEC and PDAC® cells, strongly suggest that soluble factors secreted by PDAC® cells mediate effects on endothelial cells including cell survival (and/or proliferation) under conditions of serum starvation, and cell migration and tube formation, consistent with results from mesenchymal-like cells from other sources (32, other refs). Angiogenic factors detected in media supernatants from cultured PDAC® cells included FGF-2, G-CSF, IL-8, PDGF-BB, VEGF, IL-6, MCP-1, and most prominently HGF.

HGF plays a major role in embryonic organ development, adult organ regeneration and wound healing. Furthermore, HGF is central to angiogenesis and tissue regeneration (37) and is thought to exert positive effects during the repair of vascular pathologies including stroke (38;39). HGF's role in angiogenesis includes not only induction of new blood vessel formation, but also maturation of nascent blood vessels, suggesting effects distinct from VEGF (40) and confirming the requirement for multiple soluble factors to drive the angiogenic process. Although highly expressed by PDAC® cells, HGF appears to contribute only in part to the mechanism of action of enhanced endothelial cell survival and/or proliferation, but is not sufficient to explain all of the observed effects. This is supported by our RNA silencing studies of HGF where we observed that >90% silencing of HGF in PDAC® cells resulted in only 20% inhibition of PDAC®-induced HUVEC survival, indeed implying that other factors play a role and/or are needed to convey the activity of PDAC® cells. Furthermore, the lack of significant success of HGF monotherapy in clinical trials for critical limb ischemia (41;42), also points to the importance of additional mediators. Candidate factors include VEGF and FGF-2 (40), IL-6 (34) , and IL-8 (35). These findings highlight the multiple functions that PDAC® cells may offer for the treatment of diseases that do not respond to standard therapies or to single biologics. In the mouse and rat HLI models, PDAC® cells demonstrated sustained effects beyond their observed persistence of four to seven days (REF, CHF PDAC paper). The effects also lasted longer

than the endogenous healing process that usually reaches a plateau at 21 to 28 days post surgery. Indeed, blood flow in the injured limb of mice continued to increase at 49 days after PDAC® cell administration, and histological evaluation of the injury site indicated that the numbers of both small and large blood vessels were increased in comparison to untreated controls. Furthermore, greater increases in large blood vessels were observed with PDAC treatment compared to treatment with VEGF. The secretion of high levels of HGF and modest levels of VEGF by PDAC® cells appear to contribute at least in part to the observed angiogenic effects. However, non-viable PDAC® cells were ineffective in the HLI model suggesting that PDAC® cells are not merely a delivery system for growth factors. It would be unlikely for a cocktail of factors to reproduce the diverse beneficial effects of live PDAC® cells which include the generation of new small blood vessels and support for their subsequent maturation, protection of the existing larger blood vessels in the injured tissue, as well as reduction of inflammatory infiltrates and promotion of muscle regeneration. Taken together, the data suggest that PDAC® cells exert an immediate, local angiogenic effect which triggers downstream events leading to a long-term effect on the endogenous homeostasis of the injured tissue.

The current study demonstrating the angiogenic capacity of PDAC® cells is consistent with our previous results in a rodent model of stroke (Shehadah 2014), in which PDAC® cells (administered as the i.v. formulation PDA-001) promoted endothelial cell proliferation and vascular density and perimeter in ischemic brain, and with results from other laboratories examining mesenchymal-like cells from various sources (mainly bone marrow and adipose tissue) in rodent HLI (reviewed in Liew et al., 2012). However, human placenta has unique attributes as the source of mesenchymal-like cells for therapeutic use: in addition to the ready availability of healthy, young, and abundant donor tissue from full term pregnancies, the status of the placenta as "immune privileged" (Guller 1999) may confer a possible advantage for allogeneic, "off-the-shelf" use in patients. In addition, we note that PDAC® cells were reproducibly effective at a dose of 40,000 cells/Kg I.M., which translates to a 1,000 cells per mouse or rat. Studies with MSCs from other sources in HLI models observe efficacy with I.M. doses of 10^5 to 10^7 MSCs per mouse (reviewed in Liew 2012). The reason for this disparity is not clear and may be due to differences in cell types, expansion methods or formulation.

In conclusion, we have demonstrated that PDAC cells can promote angiogenesis and tissue regeneration, suggesting that PDACs may be a promising candidate for the treatment of complex peripheral arterial diseases. PDAC cells

enhance endothelial cell survival, proliferation and migration in vitro, and promote blood vessel formation and maturation resulting in increased blood flow and vascular density, reduce inflammation, and promote muscle regeneration in animal models of ischemic injury. A live cell therapy with diverse functions, the ability to dynamically respond to environmental signals and to stimulate repair functions that last beyond their presence in vivo, PDAC treatment may be more effective than single angiogenic growth factors or gene therapy approaches. Studies in progress are aimed at elucidating the mechanism(s) of action by which this cell therapy exerts its effects.

Figure Legends

Figure 1:a) Effect of PDAC® cells on HUVEC survival. HUVEC were exposed to PDAC® cells via a transwell (PDAC® : HUVEC ~2:1). The initial value of HUVEC (after 6 hour serum starvation in EBM and prior to transwell exposure) was set to 100%. The difference between HUVEC survival in SF-DMEM overnight (~35%) and in the presence of PDAC® cells (72%) was statistically significant ($p<0.01$). n=7, error bars indicate standard deviation. b) Dose-dependent effect of PDAC®cells on HUVEC survival. n=3, error bars indicate standard deviation.

Figure 2: a) Stimulation of HUVEC migration by PDAC® cells. PDAC® cells and conditioned SF-DMEM (PDAC®-CM) stimulated ~70% greater HUVEC migration compared to SF-DMEM alone ($p<0.01$). EGM-2 was the positive control for HUVEC cell migration. n=7, error bars indicate standard deviation. b) Stimulation of network tube formation by PDAC®-CM. Representative photomicrographs of PDAC®-induced (upper panel) and SF-DMEM control (lower panel) network (tube) formation are shown. Arrows depict representative tubes.

Figure 3: Quantitation of factors secreted by PDAC® cells. Culture supernatants were analyzed as described for the indicated set of secreted factors. Of the factors examined, HGF is the most abundant, followed by MCP-1 and IL-6. n=5, error bars indicate standard deviation.

Figure 4: PDAC® cells promote vascularization. % change of blood vessel (BV) density was measured in the CAM assay. PDAC® cell-induced vascularization of the CAM was statistically indistinguishable from that of the positive control FGF-2 and significantly greater than the blood vessel density induced by either the vehicle control ($p<0.01$) or the basal medium ($p<0.01$). n=7, error bars indicate standard deviation.

Figure 5: PDAC® cell administration increases blood flow in rodent models of HLI. Blood flow was measured by non-contact laser Doppler at the indicated times post-surgery. PDAC® cells were administered at the indicated doses. Blood flow to the undamaged limb is defined as 100%. Representative experiments from duplicate studies are shown.

a) mouse model. The increase in blood flow in all PDAC® cell treatment groups compared to the vehicle control was statistically significant (from day 28 through day 49, $p<0.01$). n=15, error bars indicate standard deviation. b) rat model.

At 35 days, the difference in blood flow between PDAC® cell-treated groups and the vehicle control group is statistically significant ($p<0.001$). Non-viable PDAC® cell control was statistically indistinguishable from the vehicle control. n=12, error bars indicate standard deviation.

Figure 6: PDAC® cell-treatment increases the angioscore in rat ischemic limb. a) Representative angiographs taken at day 35 post-surgery. Rats were treated with A: Vehicle, or B: PDAC® cells (4×10^4/Kg). Arrows indicate the surgical site. b) Angioscore of rat hindlimb, 35 days after induction of ischemia. Rats were treated with the indicated doses of PDAC® cells or vehicle control; angioscore of the undamaged limb is defined as 100%. n=5, error bars indicate standard deviation.

Figure 7: Histology of cross sections of mouse quadriceps muscle. Quadriceps slices from mice (49 days after surgery) were stained with hematoxylin and eosin; representative micrographs (40x magnification) are shown. The pink shapes are muscle fibers, the white borders are connective tissue; (a) non-surgical limb, (b) surgical limb from animals treated with vehicle control, (c) VEGF control, and (d) PDAC® cells at 4×10^4 /Kg dose. Scale bar = 50 µm.

Figure 8: (a) Evaluation of capillary density in cross sections of mouse quadriceps muscle. Quadriceps slices from mice (49 days after surgery) were stained with indoxy-tetrazolium to detect alkaline phosphatase, a marker of newly-synthesized capillary endothelial cells. Representative micrographs (40x magnification) are shown for surgical limbs treated with vehicle control (left panel) or PDAC® cells (4×10^4/Kg dose) (right panel). Arrows designate representative areas of alkaline phosphatase staining. Scale bar = 100 µm. n=5. (b) Size distribution of small and large blood vessels in mouse limbs 49 days post-surgery. PDAC® cells were administered at the indicated doses. n=5, error bars indicate standard deviation.

Figures

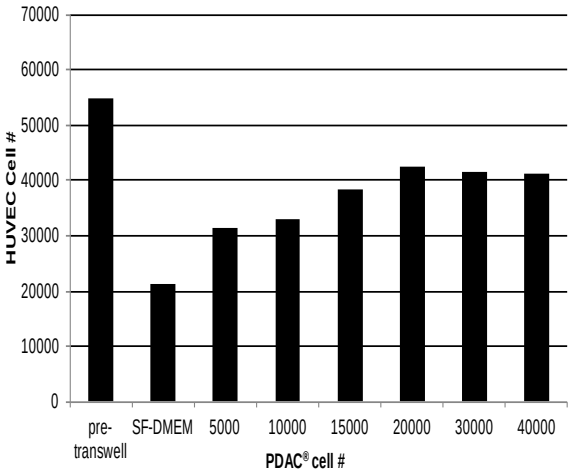


Figure 1a

Figure 1b

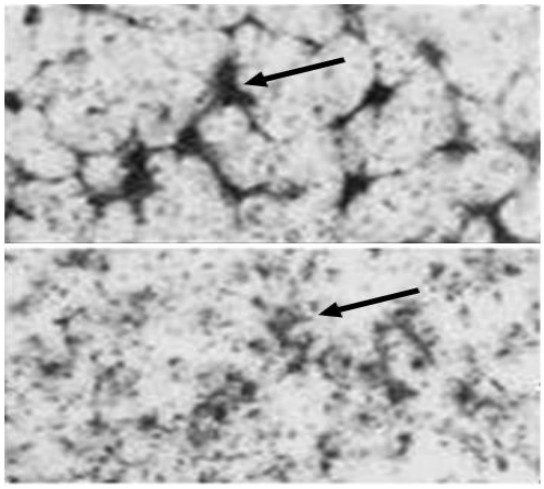
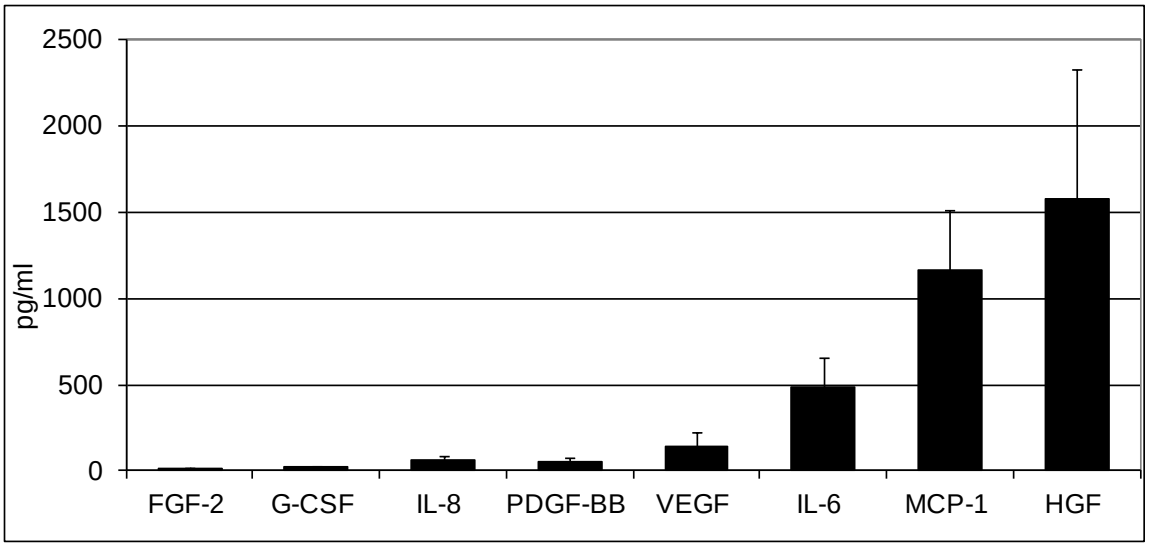


Figure 2a

Figure 2b



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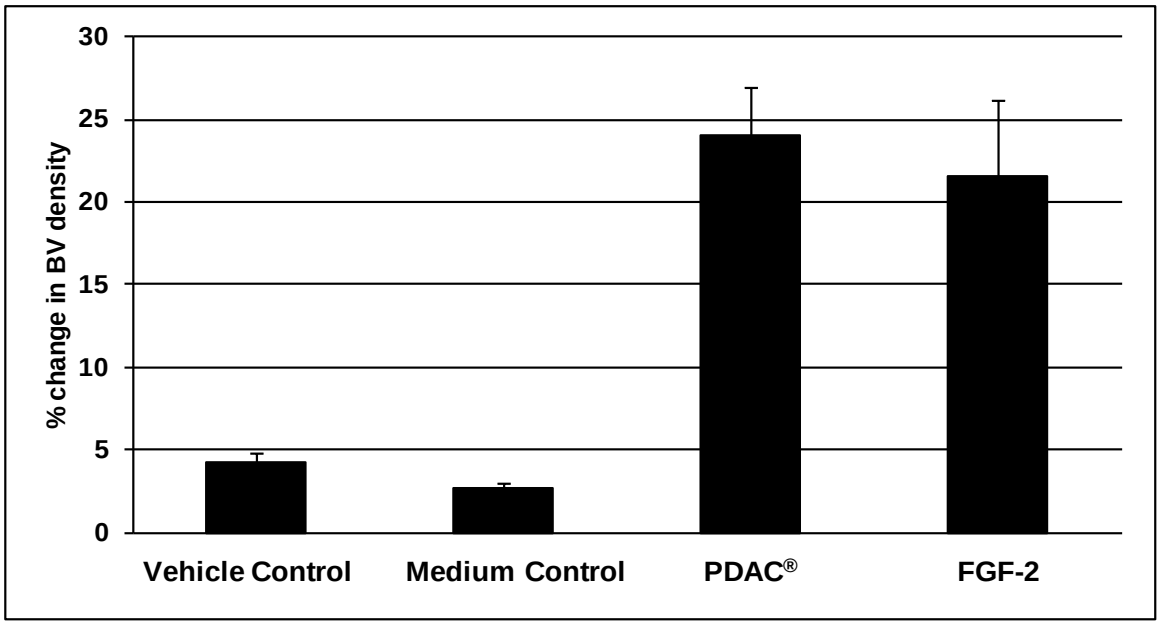
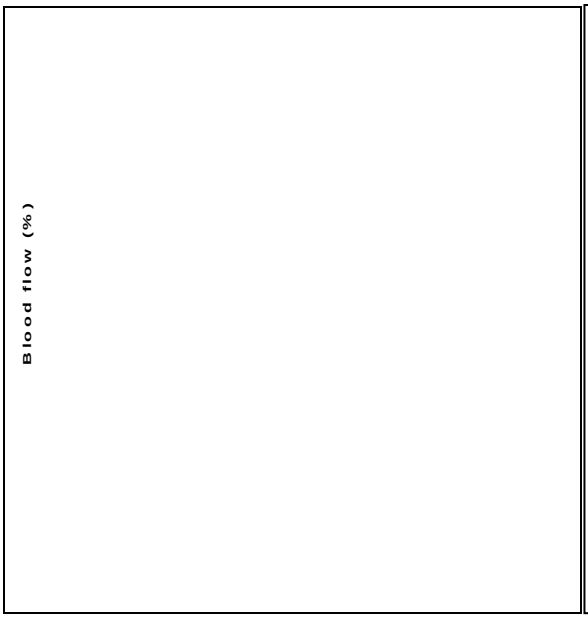


Figure 4



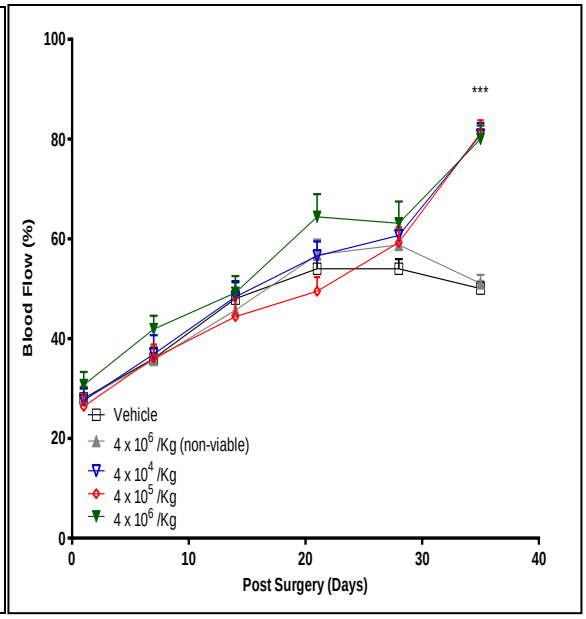


Figure 5a **Figure 5b**

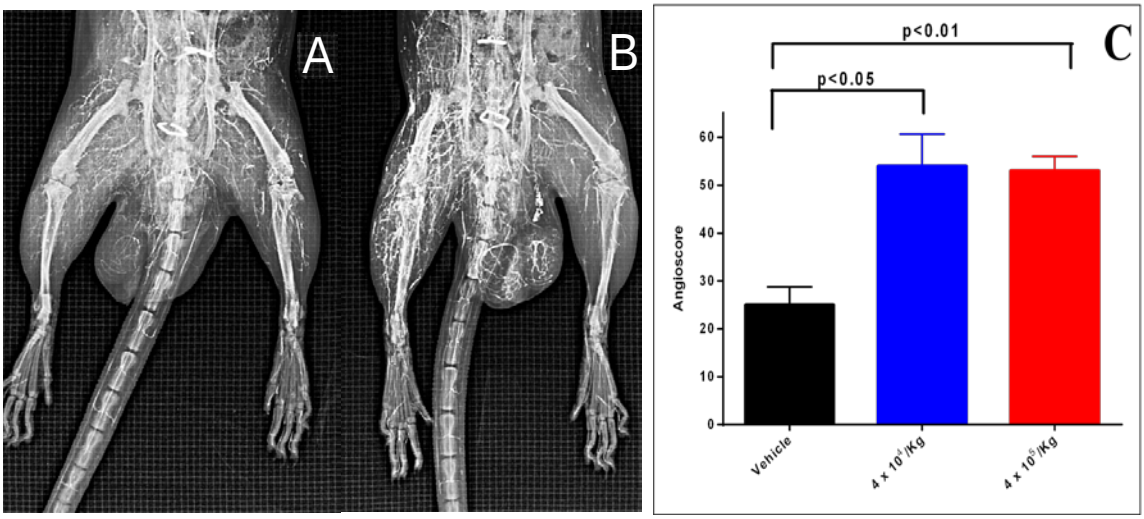


Figure 6

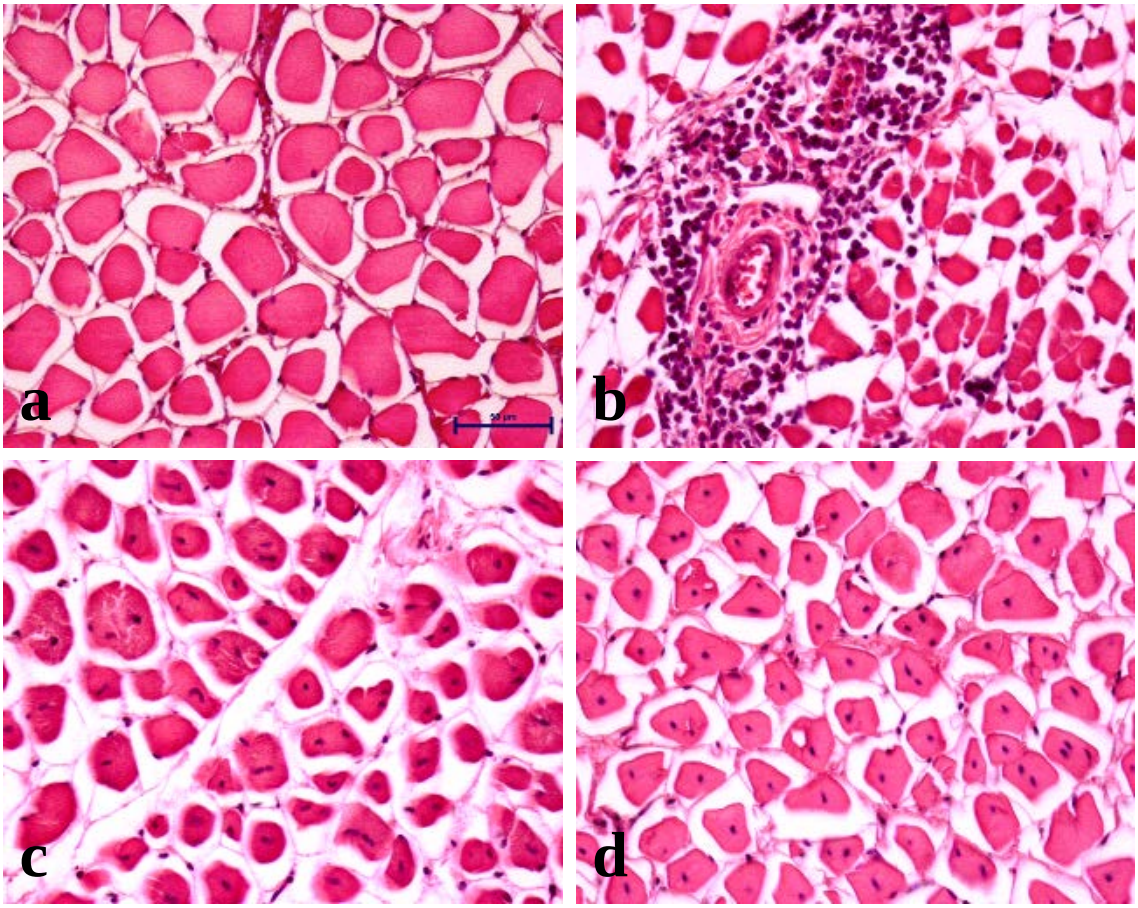


Figure 7

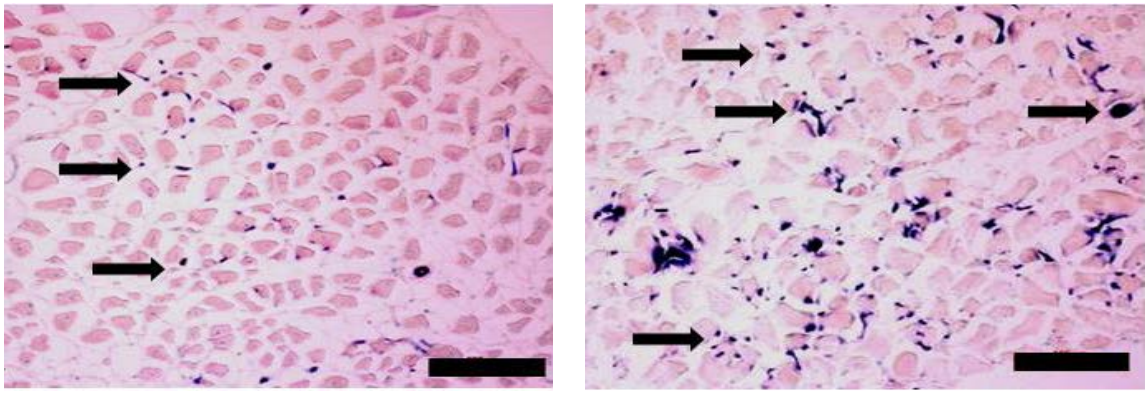


Figure 8a

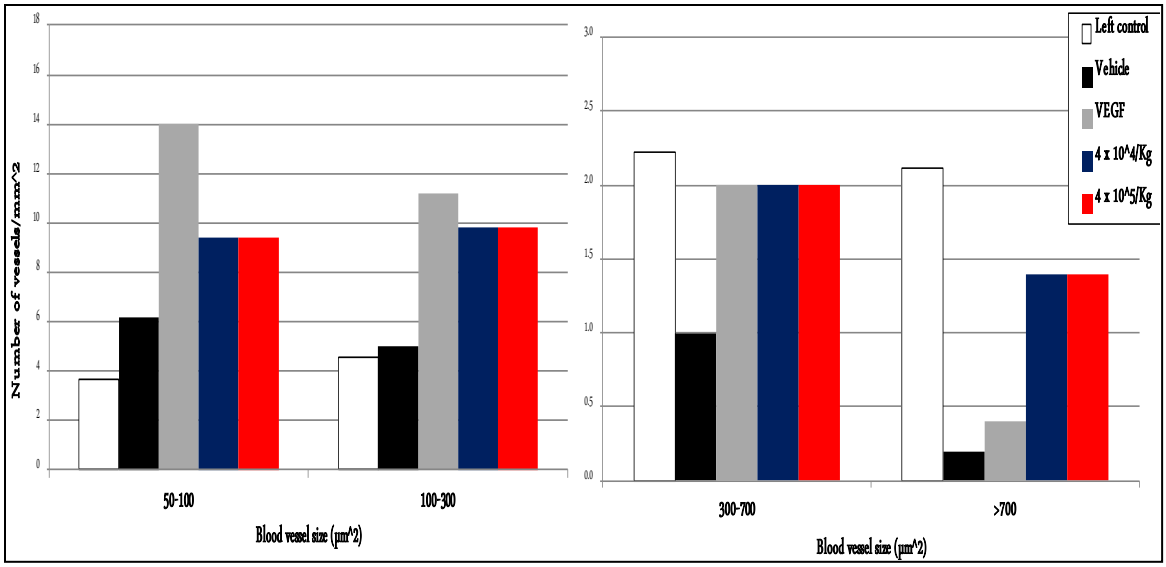

Number of vessels/mm^2
18
16
14
12
10
8
6
4
2
0
50-100
100-300
Blood vessel size (μm^2)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
300-700
>700
Blood vessel size (μm^2)
Left control
Vehicle
VEGF
4 x 10^4/Kg
4 x 10^5/Kg

Figure 8b