**SUPPLEMENTAL MATERIAL**

Materials and Methods

*Alginate Gel Formulation and Preparation*

High and low molecular weight alginates (Pronova MVG #4200106 and VLVG #4200506, respectively) were oxidized separately as described previously(1). Briefly, sodium periodate equaling 1% of the molar mass of the uronate residues was added to a 1% (w/v) alginate solution and reacted for 17 hours at room temperature. After 17 hours, an equimolar amount of ethylene glycol was added to stop the reaction. The alginate solution was dialyzed (SpectraPor MWCO 3500) against deionized water for 3 days. Following dialysis, all materials were frozen and lyophilized. For mouse studies, high and low molecular weight oxidized alginates were reconstituted with phenol red-free EMB-2 (Lonza #CC-3129) and were mixed in a 3:1 ratio (LMW:HMW), followed by addition of VEGF and IGF (R&D Systems #293-VE/CF and #291-G1, respectively) such that the final concentration of alginate was 2% w/v and 3µg of each protein was contained in 50µl of gel solution. For rabbit studies, standardized alginate kits were prepared such that one kit contained enough material to treat one rabbit. Oxidized, lyophilized alginates were reconstituted in a 3:1 ratio (LMW:HMW), sterile filtered, aliquoted into sterile containers and frozen and lyophilized again. Samples were then capped under vacuum to be ready for use. Growth factor aliquots consisted of 30µg of VEGF or 30µg of VEGF and 30µg of IGF diluted in 150µl of EBM-2, resulting in a final dose of 20µg of each growth factor per rabbit contained in 0.5ml of alginate. Calcium sulfate aliquots consisted of a 0.75M calcium sulfate slurry. To prepare alginate hydrogels for injection, lyophilized alginate was reconstituted to 2.5%w/v with EBM-2 and allowed to dissolve for 30 minutes. 600µL of alginate was removed from the vial, mixed with a 150µL growth factor aliquot to produce a final alginate concentration of 2% w/v. To crosslink gels for mouse and rabbit studies, the alginate solution was then mixed with the calcium sulfate slurry in a ratio of 25:1 (40µl of $CaSO_4$ per 1ml of 2%w/v alginate solution). Twenty minutes after mixing, gels were fully cross-linked and ready for injection. Gels were typically injected 1 hour after preparation, but material properties remain stable between 20 minutes and 24 hours.

*Rheology*

Viscosity of high and low molecular weight alginate solutions, and the combined formulation were characterized using a TA Instruments AR-G2 rheometer. 1% (w/v) solutions were prepared and tested using dual cylinder geometry at room temperature. A stress sweep from 0.1 to 8 Pa was used to test solution viscosity over a linear range.

*Gel permeation chromatography*

Initial and final molecular weights of alginates were tested using Viskotek TDA max with TDA 305 triple detector, consisting of refractive index, instrinsic viscosity, and right angle light scattering (RALS). The mobile phase consisted of filtered, 0.1M sodium nitrate and was degassed by an on-line degasser. Tosoh Biosciences G5000PWxl and G4000PWxl columns were placed in series and maintained at 35°C with a flow rate of 0.7mL/min. Alginate solutions of 1mg/mL in water were prepared, and 100µL sample volumes were injected. All chromatograms were analyzed using OmniSec software.

*In vivo gel degradation*

Alginate polymers were first fluorescently labeled with HyLite Fluor™ 750 hydrazide (Anaspec #81268) such that 1 out of every 105 uronate residues was modified with a dye molecule. Briefly, alginate polymers were transferred to 2-(*N*-morpholino)ethanesulfonic acid (MES) Buffer (100mM MES, 300mM NaCl, pH=5.0) following oxidation. 5mg/ml alginate solution was reacted with 12 molar equivalents of HyLite dye, 120 molar equivalents of N-Hydroxysuccinimide (sulfo-NHS, Thermo Scientific #24510), and 60 molar equivalents of N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, Sigma #E7750) overnight. Hydroxylamine was added to quench the reaction (1000 molar equivalents), and alginate was then dialyzed in baths of decreasing salt concentration for 4 days before sterile filtration and lyophilization.

Fluorescently labeled high and low molecular weight alginates were then made into hydrogels as described above. 50µl of alginate gel was injected into the hindlimb muscle of mice; non-labeled alginate was used as a control. Mice were then imaged with an In Vivo Imaging System (IVIS Spectrum, Perkin

Elmer) at various time points over a few months. All mouse protocols followed institutional guidelines set by the Harvard University IACUC.

*Angiogenesis Cytokine Array*

Peripheral blood samples were collected from young and middle age animals (n=5), allowed to clot at room temperature for 30 minutes, then were centrifuged at 13,000rpm at 4˚C for 10 min. The supernatant from each condition was collected and stored at -20˚C for further quantification. The relative expression levels of 53 mouse angiogenic cytokines was quantified via proteome profile array (R&D Systems #ARY015) according to manufacture guidelines and protocol (Fig S1 for array map).

*Mouse Hind-limb Ischemia Model and Blood Vessel Analysis*

All mouse protocols followed institutional guidelines set by the Harvard University IACUC.

Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg) prior to all surgical procedures. Hindlimb ischemia was induced by unilateral external iliac and femoral artery and vein ligation as previously described(1–3). Immediately after vessel ligation, an intramuscular injection of 50µl of alginate gel containing 3µg of each VEGF and IGF, VEGF alone, IGF alone, or no growth factors (control condition) were given directly into the area where the vessels were ligated. For dosing studies in old mice, animals were given 50µl of alginate gel containing 6µg or 12µg of VEGF. Incisions were subsequently surgically closed and animals monitored over time.

Mice were imaged with a Laser Doppler Perfusion Imaging (LDPI) system (PeriScan PIM II, Perimed Instruments, Ardmore, PA) prior to performing surgery, and at multiple time points up to 12 weeks post-surgery. Each animal was scored according to the level of ischemia present: no necrosis (7), one nail discoloration (6), two or more nail discoloration (5), one toe discoloration (4), two or more toe discoloration (3), foot necrosis (2), leg necrosis (1) and autoamputation (0). To quantify blood vessel density, hindlimb adductor muscle tissues were retrieved at the end of 12 weeks, fixed, paraffin embedded, and stained for mouse CD31 (BD Biosciences #557355) as previously described(1, 3). Sections were imaged on a Nikon Eclipse E800 light microscope (Japan), and the number of positively

stained blood vessels in 30 images per condition were manually counted and normalized to the tissue area.

*Muscle function quantification*

Tibialis anterior muscles were dissected intact along with their tendons (n=4 per condition), mounted vertically between two fine cylindrical parallel steel wire electrodes (1.6mm in diameter, 21mm in length), attached by their tendons to microclips connected to a force transducer (FORT 25, WPII) and bathed in a physiological saline solution in a chamber oxygenated with 95% $O_2$ and 5% $CO_2$ at 25°C as described previously(4). A wave pulse was delivered to the stimulation electrodes via a purpose-built power amplifier (QSC). Contractions were evoked in 5 min intervals and were continuously monitored. The peak of the tetanic contractile force was determined as the difference between the maximum force during a contraction and the unstimulated baseline level, and specific force was calculated by normalization to muscle wet weight as described in detail previously(4, 5).

*Rabbit Hind-limb Ischemia Model and Blood Vessel Analysis*

All rabbit procedures followed institutional guidelines set by the University of Michigan IACUC. New Zealand White rabbits (Female, 2.5-3kg) were anesthetized with a cocktail of ketamine and xylazine, and were maintained under anesthesia by isofluorane. Following an incision below the inguinal ligament and isolation of the iliac and femoral arteries and veins, ligation of the lateral circumflex artery, and the common, superficial and deep femoral arteries was performed to induce hindlimb ischemia. Ten 50µl injections of alginate gel, in total containing 20µg of each growth factor (VEGF+IGF, VEGF alone, or VEGF+IGF in saline) were then delivered in two rows of five evenly spaced locations surrounding the ligation site. A PDMS-based mold was used as a guide to ensure even spacing of injections and consistency between animals. Control rabbits were given no treatment. For one condition, rabbits were given no treatment immediately, and were then treated with VEGF+IGF containing gels on day 30 following surgery, after stable ischemia had developed. In this situation, gel was delivered via percutaneous injections using the same PDMS mold as a guide. Rabbits were monitored by Laser Doppler Perfusion Imaging (LDPI) prior to and at various time points after surgery. At the end of each experiment,

hindlimb gracilis and adductor muscle tissue surrounding the injection site was collected and formalin fixed. Muscle tissue sections, cut perpendicular to fiber direction, were stained for blood vessels using an antibody to CD31 (Abcam #ab9498). An Alexafluor-488 Goat anti-mouse secondary antibody was used for visualization (LifeSciences #A11001). Sections were imaged using a Zeiss Axio Observer (Thornwood, NY). Six or more 3mm x 3mm images from each rabbit (n = 4-6) were used for quantification.

*Growth Factor Analysis in Rabbit Serum*

Approximately 1ml of rabbit blood was collected from the ear vein in order to measure growth factor concentration in the serum. Blood was collected prior to surgery, and 3 and 24 hours after injection of alginate gels. Blood was allowed to clot for 30 minutes at room temperature and was then centrifuged at 17,000 rpm for 10 min. Serum was collected and stored at -20$^{o}$C until human VEGF and IGF concentration was analyzed in duplicate by ELISA.

*Angiography*

Angiographies were performed on two rabbits per treatment group, with one on day 28 and the other on day 49. The rabbits were anesthetized with ketamine (20mg/K) and maintained under anesthesia with isoflurane. Following catheter insertion into the common carotid artery, the catheter tip was positioned proximal to the branch point of the common iliac arteries under the guidance of a Philips Heathcare X-RAY imaging instrument (#NEMO12, Saronno, Italy). 10ml of contrast medium (270mg iodine/ml) was manually injected at a rate of 2 ml/sec. Serial images were then recorded at a rate of 6 images/sec for 6 seconds. The image representing the best arterial filling was chosen for analysis. The length of the hypogastric artery and all whole visible collateral branches were measured (Fig S5) using cellSens Dimension software (Olympus, Tokyo, Japan).

*Statistical Analysis*

All data was compared using either Students t-test or a One-way ANOVA with Tukey post hoc test; $p<0.05$ considered statistically significant.

References


1. Silva EA, Mooney DJ (2007) Spatiotemporal control of vascular endothelial growth factor delivery from injectable hydrogels enhances angiogenesis. *J Thromb Haemost* 5:590–598.

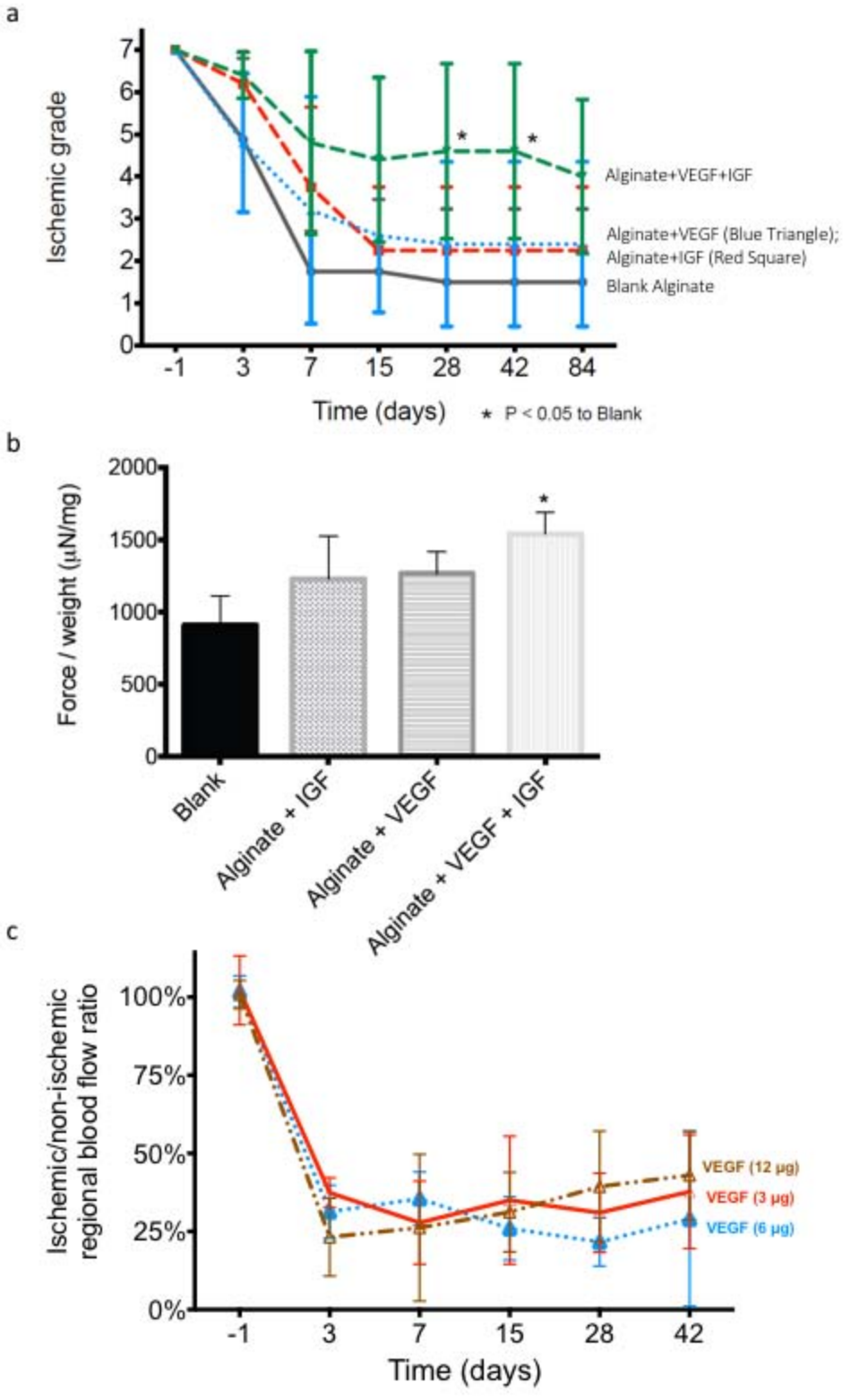
2. Chen RR et al. (2007) Integrated approach to designing growth factor delivery systems. *FASEB J* 21:3896–3903.

3. Silva EA, Mooney DJ (2010) Effects of VEGF temporal and spatial presentation on angiogenesis. *Biomaterials* 31:1235–41.

4. Borselli C et al. (2010) Functional muscle regeneration with combined delivery of angiogenesis and myogenesis factors. *PNAS* 107:3287–3292.

5. Duty S, Allen DG (1994) The distribution of intracellular calcium concentration in isolated single fibres of mouse skeletal muscle during fatiguing stimulation. *Pflugers Arch* 427:102–9.

| | 1 2 | 3 4 | 5 6 | 7 8 | 9 10 | 11 12 | 13 14 | 15 16 | 17 18 | 19 20 | 21 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Positive Control | | ADAMTS 1 (METH 1) | Amphiregulin | Angiogenin | Ang -1 | Ang -3 | Coagulation Factor III | CXCL16 | | Positive Control |
| B | | Cyr61 (CCN1, IGFBP-10) | Dll4 | DPPIV | EGF | Endoglin (CD105) | Endostatin | Endothelin-1 | FGFa | FGFb | |
| C | | KGF (FGF-7) | Fractalkine (CX3CL1) | GM-CSF | HB-EGF | HGF | IGFBP-1 | IGFBP-2 | IGFBP-3 | IL-1α | IL-1β |
| D | | IL-10 | IP-10 (CXCL10) | KC (CXCL1, Groα) | Leptin (OB) | MCP-1 | MIP-1α | MMP-3 | MMP-8 | MMP-9 | NOV (CCN3, IGFBP-9) |
| E | | Osteopontin | PD-ECGF | PDGF-AA | PDGF-AB/ PDGF-BB | Pentraxin-3 | CXCL4 (PF4) | PlGF-2 | Prolactin | Proliferin | |
| F | Positive Control | SDF-1 | Serpin-E1 (PAI-1) | Serpin-F1 (PEDF) | TSP-2 | TIMP-1 | TIMP-4 | VEGF | VEGF-B | Negative Control | |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Alginate+IGF (Red Square)
Blank Alginate
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b
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Alginate + IGF
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Alginate + VEGF + IGF
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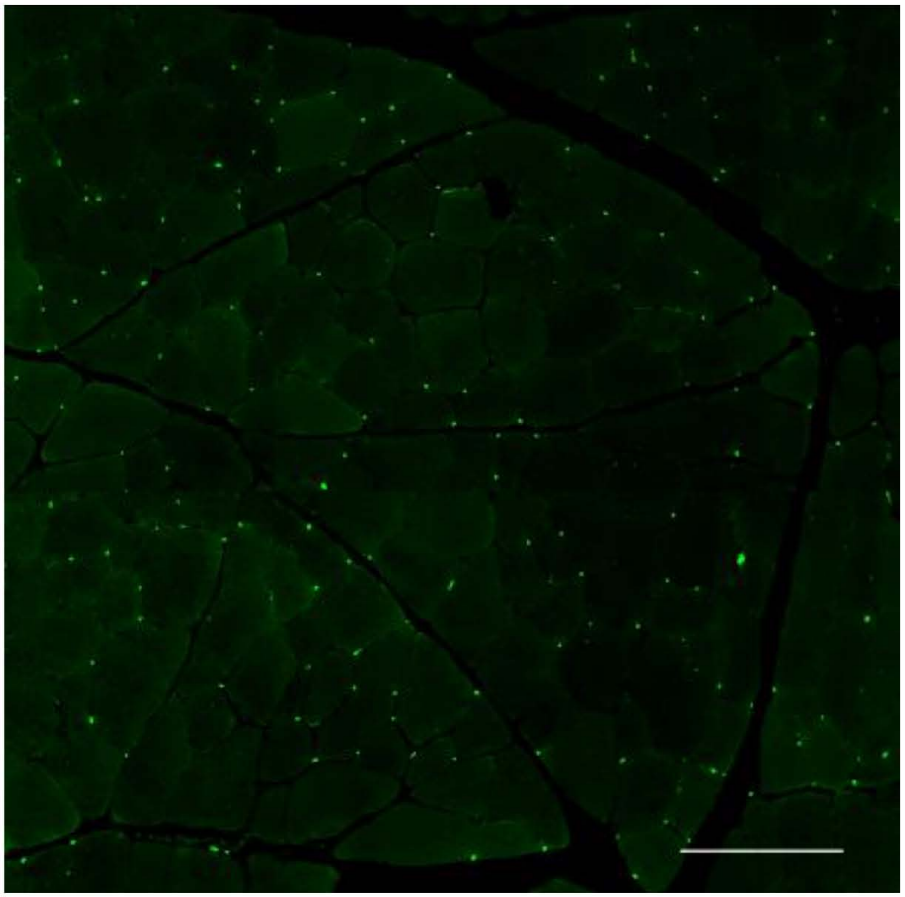
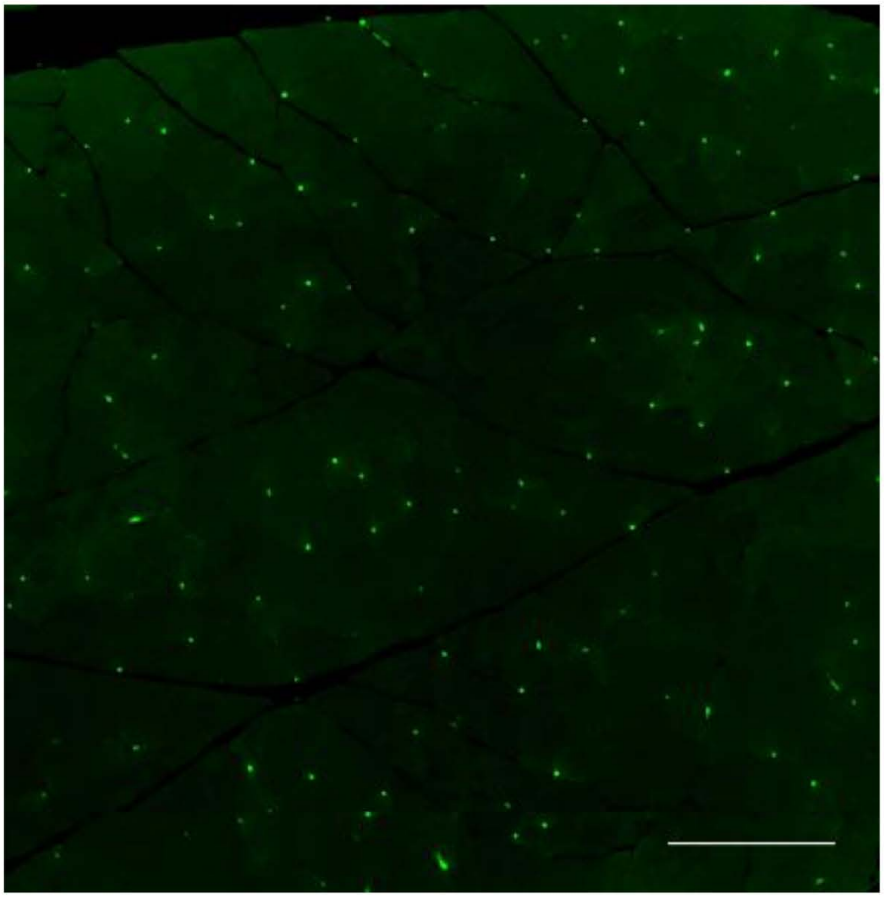


VEGF+IGF Bolus



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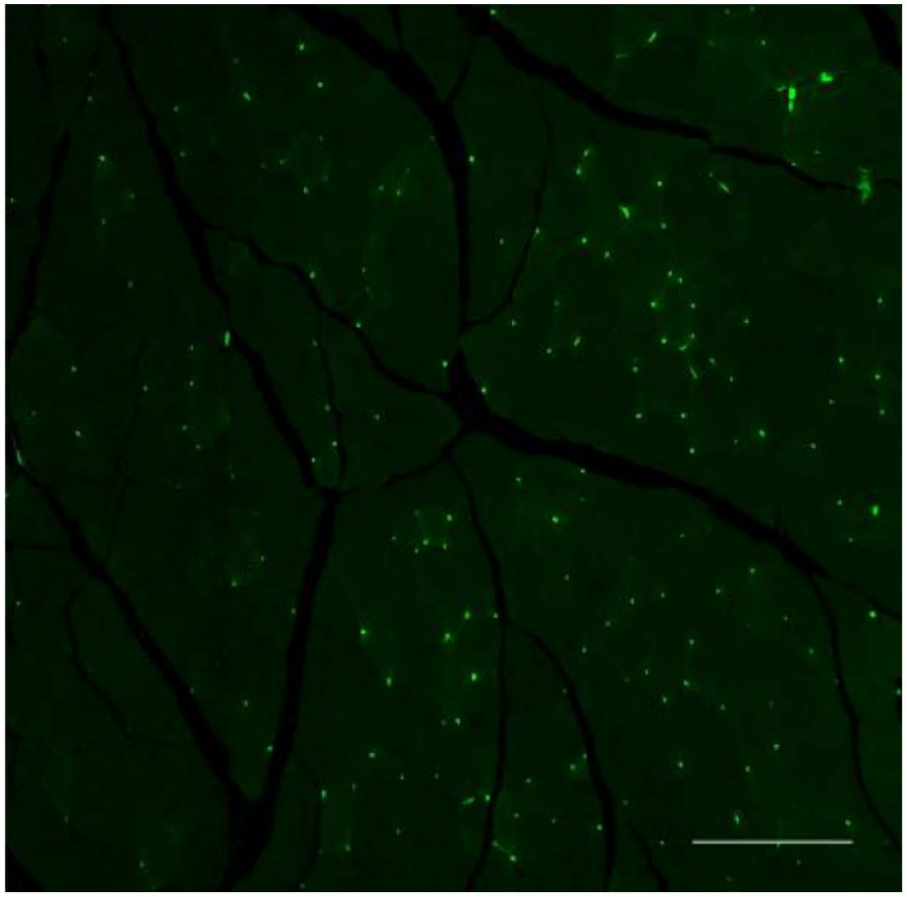
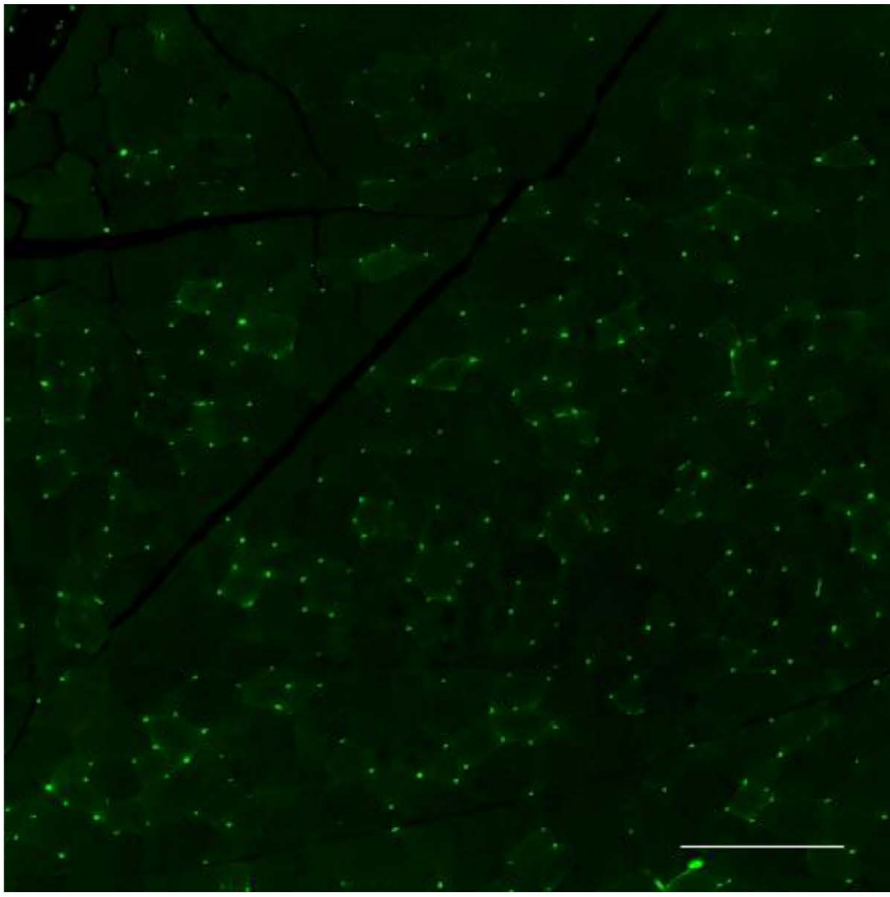
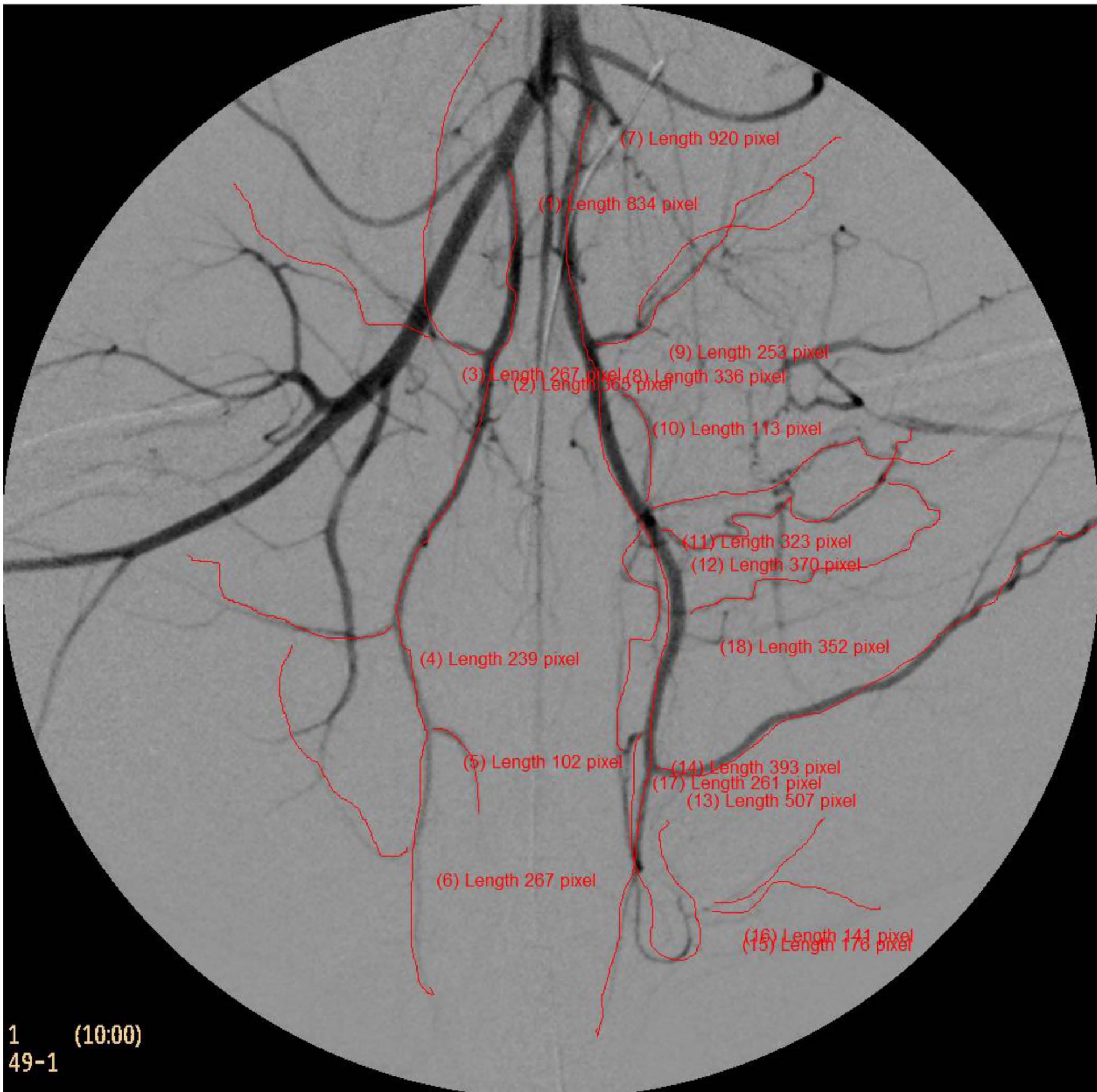


VEGF + IGF Gel

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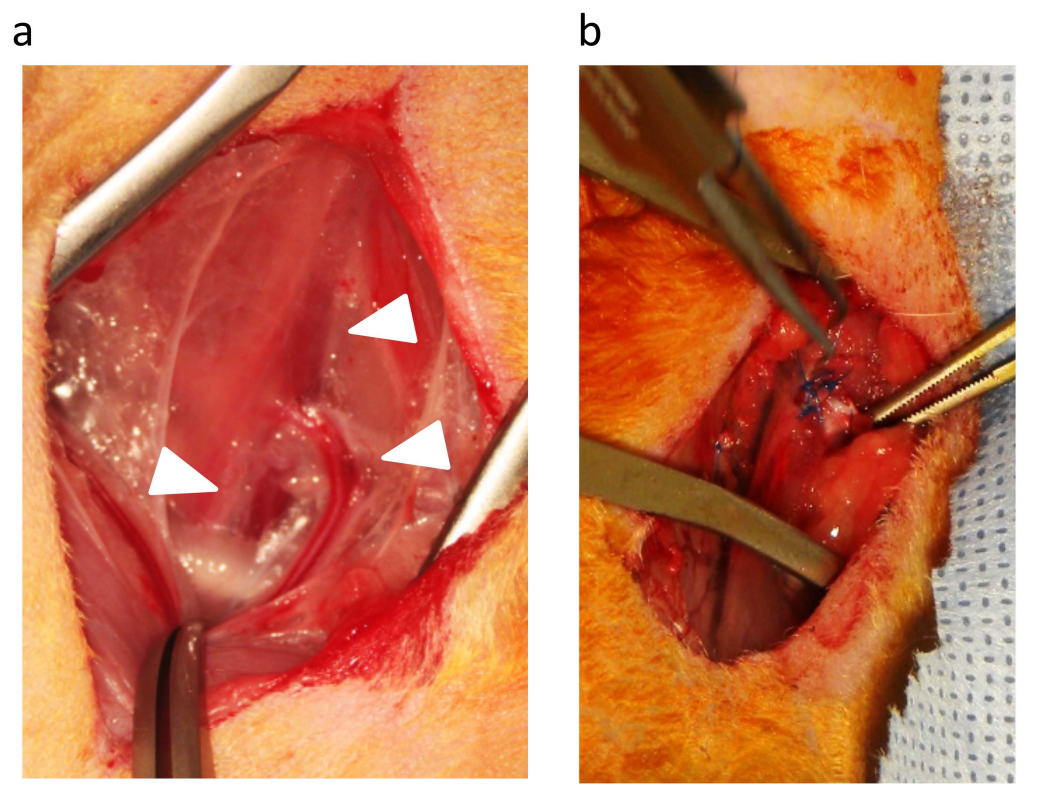
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**Supplemental Table 1. Representative dosing regimens used in previous human clinical trials and preclinical large animal studies utilizing VEGF protein delivery.**

| **Species** (# subjects) | **Delivery Route**[1] | **Application**[1] | **Dosing Regimen** | | | **Reference** |
|---|---|---|---|---|---|---|
| | | | **Total (ug)**[2] | **(ug/kg)**[2] | **time** | |
| **Human** (15) | IC | MI | 8 - 267 | 0.1 – 3.34 | 10 min each, left and right coronary arteries | Hendel RC et al. Circulation 2000; 101:118-21; Henry TD et al. Am Heart J 2001; 142:872-80. |
| **Human** (28) | IV | MI | ~668 | ~8.35 | 1-4 hours | Henry TD et al. Curr Interv Cardiol Rep 2000; 2:228-241. |
| **Human** (113) | IC +IV | MI | 1000 or 2960 | 12.58 or 37 | 20 min IC on day 0 + 4 hours IV on days 3,6,9 | Henry TD et al. Circulation. 2003; 107:1359-1365. |
| **Mini swine** (5) | IV | MI | 1200 | 30 | 200 min on days 0,3,6 | Hughes GC et al. Ann Thorac Surg 2004; 77:812–8 |
| **Mini swine** (5) | IM | MI | 600 | 15 | Single injection | Hughes GC et al. Ann Thorac Surg 2004; 77:812–8 |
| **Rabbit** (9) | IA | PAD | 500 or 1000 | 200 or 400 | Not reported | Takeshita S et al. J Clin Invest 1994; 93: 662-670 |
| **Rabbit** (8) | IV | PAD | 1000 or 5000 | 400 or 2000 | Not reported | Bauters C et al. J Vasc Surg 1995; 21:314-25. |
| **Rabbit** (17) | IM | PAD | 2000, 5000 or 10,000 | 800, 2000 or 4000 | Daily injections for 10 days | Takeshita S. et al. Circulation 1994; 90:II228–34. |
| **Rabbit** (6) | IA | PAD | 500 | 200 | Not reported | Bauters C et al. Circulation 1995; 91: 2802-2809 |

1. Abbreviations: IV – Intravascular, IC – Intracoranary, IM – Intramuscular, IA – Intraarterial, MI – Myocardial Infarction, PAD – Peripheral Artery Disease. 2. Values were calculated from dosing time and average weight.