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Fabrication of prevascularized co-culture spheroids and their 3D bioprinting application

Running title: Prevascularization for 3D bioprinting

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Abstract

While the field of tissue engineering holds great promise to address the critical shortage of tissues/organs for transplantation, a key challenge that remains is vascularizing large constructs. Presented herein is a strategy to develop a prevascular network within engineered tissues to facilitate early anastomosis with host vasculature and perfusion. A co-culture system was established that uses endothelial cells and supporting mesenchymal stem cells to generate aggregates of cells containing a network of prevascular cord and plexus-like structures reminiscent of embryonic vasculogenesis. Aggregates comprised of 50,000 cells were 537 μm in diameter, just beyond the limit of oxygen diffusion and thus appropriate for a vascularization study. Fibroblast growth factor 2 (FGF-2) was then shown to be a sufficient media supplement to enhance early vasculogenesis within the first week. A bioink of these prevascular aggregates in fibrinogen was then 3D printed into a thrombin-containing hydrogel slurry temporary support, resulting in fibrin encapsulated prevascular aggregates. These prevascular structures were shown to persist for 7 days of tissue culture. A minimum pre-printing culture time of 7 days was demonstrated to maintain prevascular structures post-printing. This system allows for the precise spatial control of prevascularized constructs that may ultimately enhance microvascularization upon transplantation in vivo.

Keywords: Tissue engineering; Prevascularization; Vasculogenesis; Aggregates; Bioink; Slurry; 3D printing

1. Introduction

While tissue engineering has great potential to alleviate the shortage of donor organs, a critical obstacle to the translational success of this technology is the need to rapidly develop a microvasculature within the engineered constructs.¹ Nearly all cells within the body lie within 150-200 μm of a capillary bed, a requirement driven by the diffusional limitation of oxygen through tissue.² Therefore, much current work in the field attempts to address this mass transport limitation by working to develop a microvascular capillary network within engineered tissues.

The endothelial cell and the pericyte, a supporting stromal cell, are two critical cells for the engineering of a functional microvascular network.³ Endothelial cells are mesodermally-derived cells that line the inner lumen of all blood vessels in the body, both macrovascular and microvascular.⁴ These cells perform many functions ranging from providing a transport barrier function to cells and large molecules to modulating intravascular coagulation.⁵ Pericytes surround capillary networks of endothelial cells, providing support for a mature phenotype and preventing leakage through the endothelium.⁶ These cells may also perform a reparative function by acting as mesenchymal stem/stromal cells (MSCs).⁷ Previous attempts at engineering a microvasculature have demonstrated greater vascular maturation via decreased leakage through the endothelium when networks of endothelial cells are formed in the presence of a stromal supporting cell such as MSCs or fibroblasts.⁸⁻¹⁰ Thus, such a cellular co-culture system may be preferable to single cell systems for microvascular tissue engineering applications.

Current co-culture systems are predominantly scaffold based approaches that utilize biomaterials to provide mechanical support and instructive signals to the cellular component as the tissue develops and act as temporary artificial extracellular matrices (ECMs). However, these systems do not allow for substantial cell-cell contact, a critical step in the development of many tissues. For instance, the vasculature in the developing embryo evolves from condensations of cells known as blood islands,¹¹ which allow for abundant cell-cell interaction and the ultimate development of both angioblasts and hematopoietic stem cells from the putative common hemangioblast precursor cell.¹¹ These cells self-assemble into prevascular cords, then plexuses and ultimately polarized capillaries with arterial and venous sides upon the introduction of blood flow. Thus, a scaffold-free cellular co-culture aggregate model of microvascular development may better mimic tissue formation through the early stages of an embryonic vasculogenesis-like process resulting in prevascular structures, ultimately forming mature vascular structures upon anastomosis and perfusion with host vascular in vivo.

Recently, systems utilizing cellular co-culture aggregates containing human umbilical vein endothelial cells (HUVECs) and mouse cord blood MSCs demonstrated cell spreading on Matrigel, diffuse laminin deposition in vitro and improved functional outcomes in myocardial infarction and hind limb ischemia models in vivo.^{12,13} However, these studies did not demonstrate development of prevascular structures in vitro which is ultimately necessary for larger scale tissue engineering. Primitive prevascular networks have been demonstrated in co-culture aggregates of HUVECs and fibroblasts or adipose tissue derived MSCs. However, these aggregates were

comprised of only a few hundred cells, resulting in dimensions well within the diffusion limit of oxygen in tissue.¹⁴

Another limitation to the fabrication of large tissue engineered constructs, in addition to the formation of a microvasculature, is the difficulty of forming spatially complex tissues in clinically relevant sizes and geometries, such as a human long bone. Advancements in 3D printing have made this technology a potentially viable solution to this problem in the field of tissue engineering.¹⁵ Cytocompatible polymer printing inks, termed bioinks, such as those based on collagen, alginate, gelatin, hyaluronic acid and poly(ethylene glycol), have been developed that allow for the printing of living cells in 3D constructs.¹⁵⁻¹⁸ Several methods of quickly stabilizing the printed constructs have been employed such as photo-, ionic-, enzymatic- or pH/thermal-induced crosslinking of macromer solutions that have been printed into a gelatin microgel slurry.^{17,19} A critical features of this microgel slurry is its shear thinning and self-healing behavior. The slurry deforms easily when the printing needle passes through it and ejects bioinks, and then quickly regains its relatively solid-like gel structure after passage of the needle so as to not allow the bioinks to flow away and maintain the 3D printed structure. The slurry may be removed via a temperature dependent sol-gel transition.¹⁷ Recently, macromer-free, cell-only suspensions and cell aggregate-only bioinks have been printed into a dual-crosslinkable alginate microgel slurry.²⁰ This microgel slurry can uniquely be further photocrosslinked to enable the printing of cell-only bioinks in complex structures and maintenance the cells in close contact with each other long enough for them to self-assemble into larger cellular condensation structures. The slurry may be designed to then hydrolytic degradation over a desired time frame.^{17,20} Despite the advances, there is limited evidence of the ability to 3D print robustly prevascularized aggregates and maintain those structures post-printing. Recently, prevascularized spheroids were 3D bioprinted in photocrosslinkable gelatin, but those cell aggregates had dimensions smaller than the diffusional limitation of oxygen and formed rudimentary vascular networks.²¹ Therefore, printing and maintaining robustly prevascularized aggregates remains a key limiting step towards 3D printing geometrically complex prevascularized constructs for tissue engineering applications.

In this study, we developed a co-culture cellular aggregate system for microvascularized tissue engineering applications. We examined the cell ratio of the aggregates and the concentration and type of growth factors they were exposed to during formation to assess their role on vasculogenic potential. The microvascularized cell aggregates were then combined with fibrinogen/alginate to create bioinks for 3D printing prevascular aggregates into a thrombin-containing alginate-based microgel slurry to create defined geometries and maintain these structures during culture. After the fibrinogen polymerized into a stable fibrin gel, the construct could be safely mechanically removed from the slurry at 1 day. The maintenance of prevascular structures during culture in the 3D printed construct was achieved for a week, thus demonstrating this system's potential utility for the construction of spatially defined prevascularized tissue engineered constructs.

2. Materials and Methods

2.1 Cell Source

Primary HUVECs (American Type Culture Collection, Manassas, VA) were expanded in endothelial growth medium-2 (EGM-2, Lonza, Basel, Switzerland), a commercial medium composed of proprietary concentrations of defined growth factors and 2% v/v fetal bovine serum, and used at passage 3. Posterior iliac crest bone marrow aspirates were obtained from healthy human donors by the Case Comprehensive Cancer Center Hematopoietic Biorepository and Cellular Therapy Core under University Hospitals of Cleveland Institutional Review Board approval (CASE 12Z05, Hematopoietic Stem Cell Facility | UH IRB No. 09-90-195) and human mesenchymal stem cells (hMSCs) were isolated from them via Percoll density gradient centrifugation followed by expansion on tissue culture plastic to select for adherent cells.²² These cells were expanded in Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM-LG) (Sigma Aldrich, St. Louis, MO) with screened 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Hyclone, Logan, UT) and a single donor was used at passage 3 for all studies.

2.2 Evaluation of cell number on co-culture aggregate formation and size

Cells were trypsinized (0.05%, Hyclone, Logan, UT), counted and suspended at a 1:1 ratio of hMSCs:HUVECs at a concentration of 250,000, 500,000, 1,250,000 or 2,500,000 cells/mL of EGM-2. To form the aggregates, 200 μ L of the cell suspension was pipetted into each well of a low adhesion, v-bottom 96 well plate (Greiner Bio-One, Kremsmünster, Austria) and centrifuged at 500 g for 5 minutes, resulting in aggregates consisting of 50,000, 100,000, 250,000 or 500,000 cells. Aggregates were then cultured at 37 °C in 5% CO₂ in a humidified incubator for the duration of the experiment with media exchanged every two days. Samples were harvested at 7 and 14 days and imaged whole mount on a TE Eclipse 300 microscope (Nikon, Tokyo, Japan) equipped with a Retiga-SRV digital camera (Qimaging, Burnaby, BC, Canada) to measure their diameter, and then they were flash frozen in Optimal Cutting Temperature (OCT) compound (Thermo Fisher, Waltham, MA) and cut into 10 μ m thick frozen sections on a HM505E cryotome (Microm GmbH, Walldorf, Germany) ($N = 4$).

2.3 Evaluation of the effect of cell ratio and culture time on prevascular structure formation

To evaluate the effect of cellular composition on the formation of prevascular structures, hMSCs and HUVECs were suspended in the following ratios in EGM-2 during the cell suspension step mentioned previously: 100:0, 90:10, 50:50, 10:90, and 0:100 for a total cell number per aggregate of 50,000. Aggregates were formed as described above and cultured for 3, 7, 10 and 14 days at which points they were flash frozen in OCT and sectioned as described above ($N = 5$).

2.4 Evaluation of the effect of exogenous growth factor delivery on prevascular structure formation

To evaluate the effect of exogenous growth factor supplementation on prevascular structures, cells were suspended in a 50:50 ratio and aggregates were formed in EGM-2 as described above. However, at the first and all subsequent feedings, the media was changed to one of the following conditions: 1) endothelial basal medium-2 (EBM-2, Lonza, Basel, Switzerland), 2) EBM-2 + 1 ng/mL FGF-2 (233-FB, R&D Systems, Minneapolis, MN), 3) EBM-2 + 10 ng/mL

FGF-2, 4) EBM-2 + 100 ng/mL FGF-2, 5) EBM-2 + 1 ng/mL VEGF (293-VE, R&D Systems, Minneapolis, MN), 6) EBM-2 + 10 ng/mL VEGF, 7) EBM-2 + 100 ng/mL VEGF, and 8) EGM-2. Aggregates were harvested at 7 and 14 days by flash freezing in OCT compound and processed on a cryostat as described above ($N = 5$). A focused version of this study was repeated with a second HUVEC donor and is reported in the Supplementary File.

2.5 Confirmation of vasculogenic potential of system with a second hMSC donor

Due to known donor-to-donor variability of hMSCs, the ability of this system to generate prevascularized aggregates was evaluated with a second hMSC donor. Optimal conditions from the initial studies were selected for evaluation. Therefore, aggregates comprised of 50,000 hMSCs and HUVECs at a 1:1 ratio were suspended in EGM-2 and formed into aggregates as before. In one experiment, these aggregates were cultured in EGM-2 for a total of 28 days, with harvests at 7, 14, and 28 days. These aggregates were then processed via frozen sections as above ($N = 5$). In another experiment, aggregates were either switched to EBM-2 with 100 ng/mL FGF-2 at 2 days of aggregate culture or maintained in EGM-2. These aggregates were harvested at 7 and 14 days of aggregate culture and processed as before for CD31 staining ($N = 5$).

2.6 Immunohistochemistry

Frozen sections were cut at 10 μ m thickness using a Microm HM 505E Cryostat (GMI Inc., Franklin, IN). Slides were stained for CD31 using a monoclonal primary anti-CD31 antibody (M0823, Agilent Dako, Santa Clara, CA) and a CINTec Histology kit (Roche, Basel, Switzerland) for colorimetric detection according to the manufacturer's instructions. Briefly, slides were warmed to room temperature (RT) for 10 minutes and then fixed in acetone for 20 minutes. The slides were then washed for 5 minutes in wash buffer from the CINTec kit to remove residual OCT, blocked with peroxidase blocking buffer, washed for 5 minutes again, incubated at RT with a 1:100 dilution of primary antibody in phosphate buffered saline (PBS) for 1 hour, washed for 5 minutes again, incubated for 30 minutes with visualization reagent (polymer reagent conjugated with horseradish peroxidase and affinity purified goat anti-Mouse Fab' antibody fragments, supplied in stabilizing solution comprising preservatives and stabilizing protein, both from the Roche CINTec Histology Kit, Catalog #06594441001), washed twice for 5 minutes, incubated for 10 minutes with substrate reagent (25-30 μ L of 3,3'-diaminobenzidine chromogen solution added to 2 mL of substrate buffer solution, pH 7.5, containing < 0.1% hydrogen peroxide, stabilizers and enhancers), washed for 5 minutes with tap water, counterstained for 2 minutes with Mayer's hematoxylin (Thermo Fisher, Waltham, MA), briefly rinsed with tap water, and then cover slips were mounted with GVA Aqueous Mounting Medium (Genemed Biotechnologies, San Francisco, CA). Negative controls were processed as described with the primary antibody omitted and PBS used instead. Samples were imaged on an Olympus BX61VS microscope (Olympus, Center Valley, PA) with a Pike F-505C VC50 camera (Allied Vision Technologies, Stadtroda, Germany).

To stain for laminin, slides were warmed to RT for 10 minutes, fixed in acetone 20 minutes and then washed in wash buffer for 5 minutes. Slides were then blocked with 1% bovine serum albumin (Sigma Aldrich, St. Louis) for 30 minutes and then incubated with primary polyclonal anti-laminin antibody (ab11575, Abcam, Cambridge, United Kingdom) at a 1:200 dilution in PBS

for 1 hour. Slides were then washed in CINTec kit wash buffer for 5 minutes. They were then incubated in an AlexaFluor 488 labeled secondary antibody (111-545-144, Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100 in PBS to visualize the laminin. Finally, after washing in wash buffer for 5 more minutes, slides were incubated 10 minutes in 2 $\mu\text{g/mL}$ DAPI (Thermo Fisher Scientific, Waltham, MA) diluted in water. Coverslips were mounted with Fluoromount (Sigma Aldrich, St. Louis, MO), and samples were imaged on the same instrument as for whole mount samples before but using fluorescent filters.

2.7 Quantification of vascular structures

Images of CD31-stained sections of prevascular aggregates were manually analyzed in ImageJ (NIH, Bethesda, MD) by a blinded scorer. Total aggregate section area was calculated, and all prevascular structures were measured. Vessels were defined as two or more CD31 positive cells that were touching end-to-end, starting and ending from either a cell touching only a single other CD31 positive cell or a junction. A junction was defined as any point where three or more vessels came together. A network was defined as either a group of vessels connected by junctions or an individual vessel not connected to junctions. From this data, total network length/area, average network length, number of networks/area, and number of junctions/area were calculated and plotted (N = 5 samples per group, 1 representative section analyzed per sample).

2.8 Preparation of alginate microgel slurry

Alginate (Manugel GMB, FMC Biopolymer, Philadelphia, PA) was purified by dialysis against Type 1 Milli-Q water using a dialysis membrane (molecular weight cut off 3500 Da, Spectrum Laboratories Co., Rancho Dominguez, CA) for 3 days with 3 water changes per day to remove impurities. This solution was then sterile filtered (0.22 μm filter) and lyophilized for a week. This purified alginate was then used to form microgels for the slurry as described before.²⁰ Briefly, the alginate (1.5g) was dissolved in DMEM-LG (100 mL), loaded into a syringe, and then dispensed into an aqueous solution of CaCl_2 (1 L, 0.2 M) to ionically crosslink and form hydrogel fibers. After the resulting alginate fibers were allowed to fully crosslink in the bath for 30 minutes, they were collected, washed with DMEM-LG three times, and then blended using a consumer-grade blender (Osterizer MFG, at “pulse” speed) for 90 seconds in 100 mL of DMEM-LG. Then, the blended alginate microgels were loaded into 50 mL conical tubes and centrifuged at $2000\times g$ for 5 minutes. The supernatant was removed and replaced with 70% ethanol. The slurry was resuspended by vortexing and centrifuged again. After the supernatant was removed, 70% ethanol was again added and the alginate microgel slurry was vortexed back into suspension and stored at 4 °C until use.

On the day of printing, the suspension of alginate microgels in 70% ethanol was centrifuged at $2000\times g$ for 5 minutes and the supernatant was removed. The slurry was resuspended in DMEM high-glucose (DMEM-HG) to a total volume of 40 mL and centrifuged again. This DMEM-HG wash was repeated three times. Human thrombin (EMD Millipore) was dissolved in Dulbecco’s phosphate buffered saline (DPBS) at a concentration of 400 U/mL and stored at -20 °C until use. On the day of printing, 25 μL of thrombin solution was added to 5 mL of DMEM-HG for a final

concentration of 4 U/mL thrombin. After the final slurry wash in DMEM-HG, 10 mL of slurry was resuspended in 5 mL of thrombin solution. This was then used for 3D printing.

2.9 Characterization of the morphology and size of alginate slurry microgels

To evaluate the morphology and measure the size of the alginate microgels that comprise the slurry, the alginate microgels were prepared as described above. After final washing, approximately 0.5 mL of slurry was mixed with 0.5 mL of 1% Safranin-O solution for 5 minutes to stain the microgels for visualization. The stained microgels were washed in 1 mL of DMEM-HG, centrifuged and then resuspended in 1 mL of DMEM-HG. Approximately 250 μ L of this washed microgel suspension was then pipetted onto a glass slide, a cover slip was added, and the microgels were imaged on the Eclipse TE300 microscope and MU1402-NI05 digital camera (AmScope, Irvine, CA) at 100 $\times$ magnification. Using ImageJ, the size of the microgels were traced and measured. Since the microgels were not spherical in shape, a long and short diameter were measured for each microgel and averaged. A total of 313 microgels were measured. The distribution of sizes was reported in a histogram and the mean value and standard deviation were also reported.

2.10 Preparation of bioinks

Human fibrinogen (EMD Millipore) was dissolved in sterile PBS at a stock concentration of 25 mg/mL and stored at -80 $^{\circ}$ C until use. To prepare the fibrinogen only bioink, 200 μ L of fibrinogen stock was mixed with 800 μ L of DMEM-HG, resulting in a final fibrinogen concentration of 5 mg/mL.

Alginate (LF20/40, FMC Biopolymer, Philadelphia, PA) was purified by dialysis, sterile filtered and lyophilized as described above. This alginate was dissolved at 2 % w/v in sterile DMEM-HG overnight to produce the alginate stock solution. To make the alginate-fibrinogen bioink, 800 μ L of 2% w/v alginate stock was mixed with 200 μ L of fibrinogen stock, resulting in a final combined solution of 1.6% w/v alginate and 5 mg/mL fibrinogen.

2.11 Measuring rheological properties of the alginate slurry and bioinks

The alginate microgel slurry and bioinks were evaluated via dynamic rheological testing to measure shear-thinning, self-healing and rheological properties on a Kinexus Ultra+ rheometer using rSpace for Kinexus software (Malvern Panalytical, United Kingdom). The slurry and bioinks were evaluated via parallel plates with a functionally infinite bottom plate and a 25 mm or 8 mm, respectively, with both operating with a 1.0000 mm working gap. Testing was performed at 25 $^{\circ}$ C for all samples, with N=3 independent replicates per group. A slurry sample was transferred to the testing platform with a spatula, and the excess was trimmed away after the platen was lowered into place.

First, a shear rate ramp was performed, in which the shear rate was varied from 0.1 to 100 s^{-1} over a 2 minute period and the viscosity was measured. This allowed for determination of shear-thinning behavior as the shear rate increased. Second, a shear stress ramp was performed from 0.1 to 100 Pa over 1 minute and viscosity was recorded to determine the shear yield stress and yield viscosity of the sample. Oscillatory strain sweep (0.1-100 % strain ramp at 1 Hz) tests were performed to show fluid-like behaviors of the OMA microgel slurries. The crossover of these

moduli, that is when G'' exceeds G' , is the point at which the phase of the viscoelastic material is taken to change from predominantly solid-like to predominantly liquid-like. Oscillatory frequency sweep (0.01-1.3 Hz at 1 % strain) tests were performed to measure storage moduli (G') and loss moduli (G'') and evaluate the stability of the slurry. The final test was a custom designed sequence to evaluate the self-healing behavior of the slurry samples. The strain was cycled between 1 and 100% at 1 Hz every minute while the moduli were recorded. Four cycles each of low and high strain were applied to evaluate the ability of the moduli to return to the same level.

2.12 3D printer modification

A PrinterBot Simple Metal 3D printer (PrinterBot, Lincoln, CA) was chosen for these studies as an established modification to the system enables use of a syringe-based extruder instead of the default thermoplastic extruder as previously described.^{17,19} This allows for 3D printing of polymer solutions of living cells. Briefly, the stock thermoplastic extruder was replaced with a custom-built syringe pump extruder assembly that was printed by the original thermoplastic extruder using polylactic acid prior to replacement. The syringe pump extruder utilized the NEMA-17 stepper motor from the original thermoplastic extruder, and both were mounted on the x-axis carriage. By using the original stepper motor, the syringe pump extruder modified PrinterBot was natively supported by the software that came loaded on the printer. The design for the syringe pump extruder was downloaded as Standard Triangle Language (STL) files from the NIH 3D Print Exchange (<http://3dprinting.nih.gov>) under an open-source license.

2.13 3D printing of aggregates into the hydrogel slurry

On the day of printing, the slurry and bioinks were prepared as described above. The 3D printer was sprayed with 70% ethanol, placed in a biosafety cabinet and irradiated with UV light for 45 minutes prior to use. The 3D printing was run on Cura 15.04.6 software (Ultimaker, Utrecht, Netherlands). For printing cubes, experiments utilized dimensions of $3 \times 3 \times 3 \text{ mm}^3$. For printing the letter 'C', the diameter of the 'C' was set at 8 mm. The aggregates to be printed were transferred via a wide-orifice pipet into a conical tube, and the media was aspirated off. The aggregates were then resuspended in the appropriate bioink at 40 aggregates per 100 μL of ink and drawn into a 1.0 mL gastight syringe (Hamilton Company, Reno, NV), and air bubbles were carefully removed from the syringe. Unless otherwise noted, the needle used for drawing the aggregates into the syringe as well as for the actual 3D printing process itself was an 18G, 0.5 inches long, blunt end needle (Component Supply Co, Sparta, TN). The syringe was loaded into the printer and secured into place. The slurry was transferred into a 24 well plate to a depth of approximately 1 cm, and air bubbles were removed by vigorously tapping the 24 well plate on the hood working surface. The extruder was manually advanced until bioink with aggregates was observed exiting the needle. At this point, the needle was lowered approximately 5 mm into the slurry and the bioink was printed. After all samples had been printed, the plate was placed into a humidified incubator (37 $^{\circ}\text{C}$, 5% CO_2) for 60 minutes to allow the thrombin to diffuse into the bioink and polymerize the fibrinogen into a gel. After the incubation period, the periphery of the slurry was aspirated, and 1 mL of media was added to the well. This media was experiment specific and was the same as what

the aggregates had been cultured in before printing. One day after printing, sterile spatulas were used to carefully release the printed constructs from the slurry. One day later (thus, 2 days after printing), a full media change was performed, during which, the slurry was also aspirated carefully to not disturb the printed construct. Feedings continued every other day for the duration of the experiment.

2.14 Comparing bioinks for 3D printing experimental set up

Two different bioinks were utilized to compare the ability to print aggregates using each ink, as well as to evaluate the inks' ability to support prevascular aggregate culture and maintenance of prevascular structures. Aggregates were prepared as described at 50,000 cells per well in EGM-2. At day 2, media changes for one group of aggregates were continued with EGM-2 while for another group of aggregates, the media was switched to EBM-2 supplemented with 100 ng/mL of FGF-2. At 7 days, these aggregates were utilized for 3D printing. Control aggregates that were not printed were harvested at 7 and 14 days, while the printed constructs were harvested 7 days after printing (thus, 14 days after aggregate formation). Groups of aggregates cultured in EGM-2 or EBM-2 with 100 ng/mL FGF-2 were resuspended in either alginate-fibrinogen bioink or fibrinogen only bioink. All harvests of control aggregates were performed in OCT as described. All harvests of printed constructs were also performed in OCT, but instead of immediate freezing in liquid nitrogen, samples were allowed to equilibrate with the OCT at 4 °C for 1 hour prior to freezing in liquid nitrogen. This allowed for diffusion of some OCT into the printed gels and osmosis of some water out of the gels, which facilitated cryosectioning. CD31 immunohistochemistry was performed by using a CINTec histology kit (Roche, Basel, Switzerland) with a mouse anti-human CD31 primary antibody (Agilent Dako, Santa Clara, CA). Briefly, slides were warmed to RT for 10 minutes, then fixed in acetone for 20 minutes. They were then washed with wash buffer from the kit and blocked with peroxidase blocking reagent. After washing with wash buffer again, they were incubated in a humidity chamber for 1 hour with a 1:100 dilution of the primary antibody in PBS. After another washing, visualization reagent was added for 30 minutes, followed by 2 more washes. DAB substrate was added for 10 minutes and slides were then washed in diH₂O. Slides were then counter stained with Mayer's hematoxylin (Thermo Fisher Scientific, Waltham, MA) for 2 minutes and washed with tap water. Coverslips were mounted with GVA aqueous mounting medium and allowed to dry overnight. They were sealed with nail polish on the next day and imaged on an Eclipse TE300 microscope with an MU1402-NI05 digital camera at 100x magnification.

2.15 3D printing different shapes and with different gauge needles experimental set up

Aggregates were prepared as described at 50,000 cells per well in EGM-2. All media changes were made with EGM-2. After 7 days, aggregates were harvested and suspended in alginate-fibrinogen bioink for 3D printing as described above. Aggregates were printed in either the shape of a 3x3x3 mm cube or an 8 mm diameter letter 'C' using an 18G needle. Other aggregates were printed through a 20G needle in the shape of a 3x3x3 mm³ cube. Control aggregates that were not printed were harvested at 7 and 14 days, while the printed constructs were harvested 7 days after printing. All samples were processed via frozen sectioning as described in

the previous section. CD31 immunohistochemistry was performed with a CINTec kit and Mayer's hematoxylin counterstaining as described above.

2.16 3D printing aggregates prior to prevascular structure formation

Aggregates were prepared as described above in EGM-2. These aggregates were suspended in alginate-fibrinogen bioink and then 3D printed with an 18G needle as described above after 2 days of culture. Controls aggregates that were not printed were either maintained in EGM-2 or switched to EBM-2 plus 100 ng/mL of FGF-2 for the duration of the experiment, and printed constructs were cultured in either of those two media as well. Controls and printed samples were harvested at 7 and 14 days post-aggregate formation and processed via frozen sectioning as described above. CD31 immunohistochemistry was performed with a CINTec kit and Mayer's hematoxylin counterstaining as described above.

2.17 Statistical Analysis

All statistics are presented as mean $\pm$ standard deviation. Tests for statistical significance involving the size and prevascular structure formation in the aggregates were performed via one-way analysis of variance (ANOVA) in Origin 2016 software (OriginLab Corp, Northampton, MA) with Tukey post-hoc test. Tests for statistical significance involving the bioink formulations were performed using a matched two-way ANOVA full model with Geisser-Greenhouse correction in GraphPad Prism 11.0.0. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Evaluation of cell number on co-culture aggregate formation and size

Co-culture aggregates were formed consisting of 50,000, 100,000, 250,000 and 500,000 cells to examine the role of cell number on aggregate formation and size. These aggregates were harvested at 7 and 14 days and stained for CD31 to identify endothelial cells that may be contributing to potential prevascular structures (Fig. 1). Grossly, the majority of cells in the two groups with less cells coalesced into a single aggregate (Fig. 1a-d), while the two larger groups (Fig. 1e-h) rarely coalesced into a single aggregate but rather frequently formed one main aggregate and several loosely attached aggregates. This phenomenon was noted throughout the duration of the experiment. At harvest, the aggregates were first imaged on a microscope to measure their intact diameter (Fig. 1i). Immunohistochemical staining for CD31 demonstrated the presence of prevascular structures in aggregates comprised of 50,000 (Fig. 1a, b) and 100,000 (Fig. 1c, d) cells at both 7 and 14 days. Limited prevascular structures were seen in aggregates comprised of 250,000 (Fig. 1e, f) or 500,000 (Fig. 1g, h) cells, although some were occasionally seen in the smaller satellite aggregates formed by cells that did not incorporate into the main aggregate (for instance, see Fig. 1f, upper right of image). Predictably, aggregates comprised of greater cell numbers were significantly larger, even if qualitatively, it appeared that a smaller fraction of cells successfully incorporated into the aggregates at higher cell number.

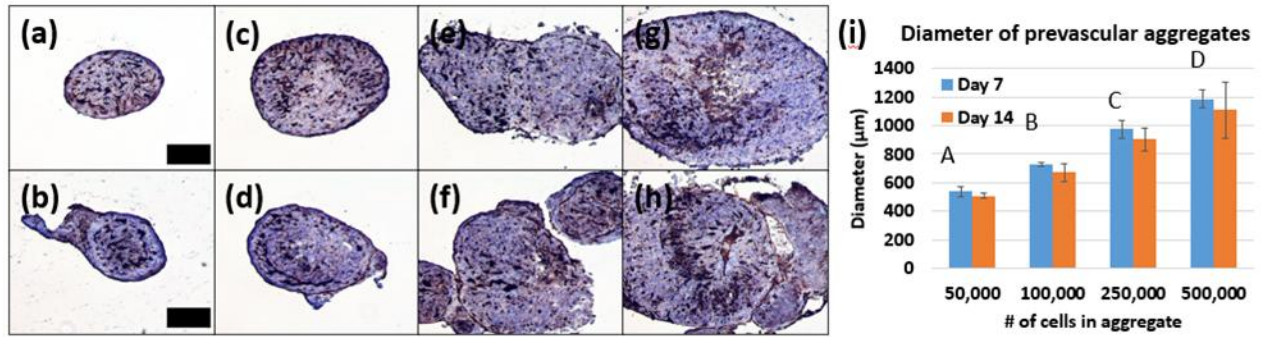


Figure 1. Effect of cell number on prevascular structure formation and aggregate diameter. CD31 staining at (a, c, e and g) 7 and (b, d, f and h) 14 days of aggregates composed of (a, b) 50,000, (c and d) 100,000, (e and f) 250,000 or (g and h) 500,000 cells showed prevascular structure formation in (a, b, c and d) smaller aggregates but poor consolidation of cells into a single aggregate at (f and h) larger cell numbers. (i) Whole mount imaging of aggregates composed of 50k, 100k, 250k or 500k cells was used to measure their diameters. Scale bars indicate 200 μ m. Cell number groups that don't share a letter were statistically significantly different by one-way ANOVA.

3.2 Evaluation of the effect of cell ratio and culture period on prevascular structure formation

The next set of experiments varied the cell ratio of hMSCs to HUVECs and the culture period to establish values for robust prevascular structure formation. Cell aggregates were made of 50,000 cells since that size was shown to form reliably and allow for prevascular structure formation. Cell aggregates composed of 0:100 and 10:90 hMSCs:HUVECs ratios did not form aggregates reliably. Instead, the cells formed many small aggregates scattered throughout the bottom of the well (data not shown). Aggregates composed of 50:50 hMSCs:HUVECs or higher hMSC ratios reliably formed a single main aggregate in the bottom of the well. A few other small aggregates were occasionally seen in the bottom of the wells in this 50:50 ratio group as well.

First, immunohistochemical staining of CD31 on endothelial cells demonstrated the formation of prevascular structures such as vessels and networks in groups containing HUVECs, but not in the 100:0 hMSCs:HUVECs group (Fig. 2a), which had no exogenously added HUVECs. Secondly, samples were stained for the basement membrane protein laminin, since basement membrane production is a marker of vascular maturation. All groups demonstrate cord-like laminin staining as opposed to a diffuse staining throughout the aggregate (Fig. 2b). Some laminin structures were observed formed in the shape of irregular rings with DAPI stained nuclei within the ring structures, however, some show ring-like laminin structures with no nuclei present within the interior of the ring (Fig. 2b). Qualitatively, the staining appeared most pronounced in the 50:50 group and in time points beyond 3 days.

The effect of culture time and aggregate cellular composition on prevascular structure formation was evaluated. The total network length/area of the prevascular structures was significantly greater in the 50:50 and 90:10 groups than the 100:0 group by days 3 and 7, respectively, and significantly greater in the 90:10 group than the 100:0 group by day 7, and remained so for the duration of the experiment (Fig. 3a). The average network length was

significantly greater in the 90:10 and 50:50 groups compared to the 100:0 group at all time points (Fig. 3b). The total network density as measured by prevascular networks per area mirrored the total network length per area results in that it was significantly higher for the 50:50 and 90:10 groups compared to the 100:0 group by days 3 and 7, respectively, and significantly higher in the 90:10 group than the 100:0 group by day 7, and remained so for the duration of the experiment (Fig. 3c). A junction is defined as a point with at least 3 CD31 positive branches emanating from it. The prevascular junction density was shown to be higher in the 50:50 group than in the 100:0 group at days 7 and 14 and higher than the 90:10 group at day 14 (Fig. 3d).

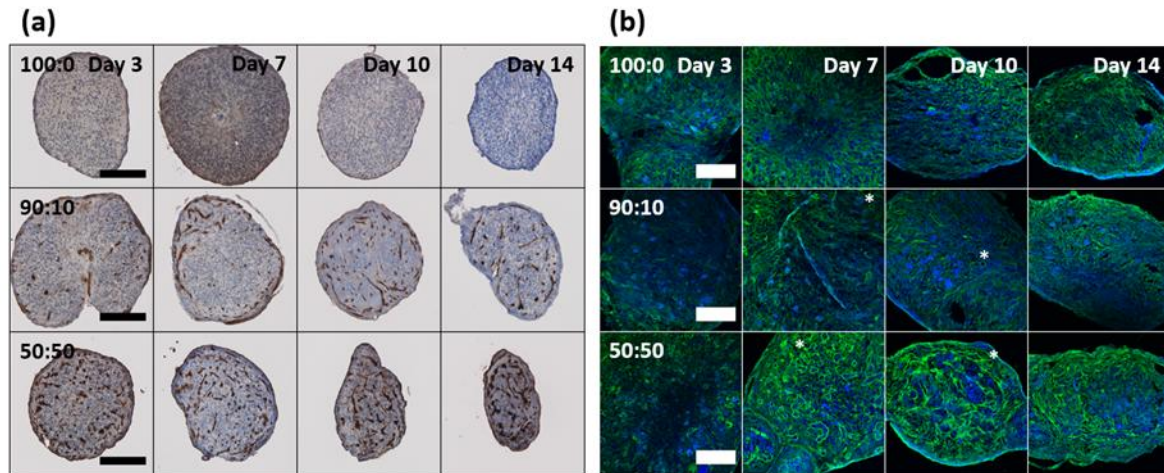


Figure 2. Time course of prevascular structure formation in co-culture aggregates of 50,000 cells with MSC:HUVEC percentage compositions of 100:0, 90:10 and 50:50. (a) CD31 and (b) laminin staining demonstrate the formation of prevascular structures and prevascular maturation respectively at 3, 7, 10 and 14 days of culture in EGM-2. Black scale bars indicate 200 μm and white scale bars indicate 100 μm .

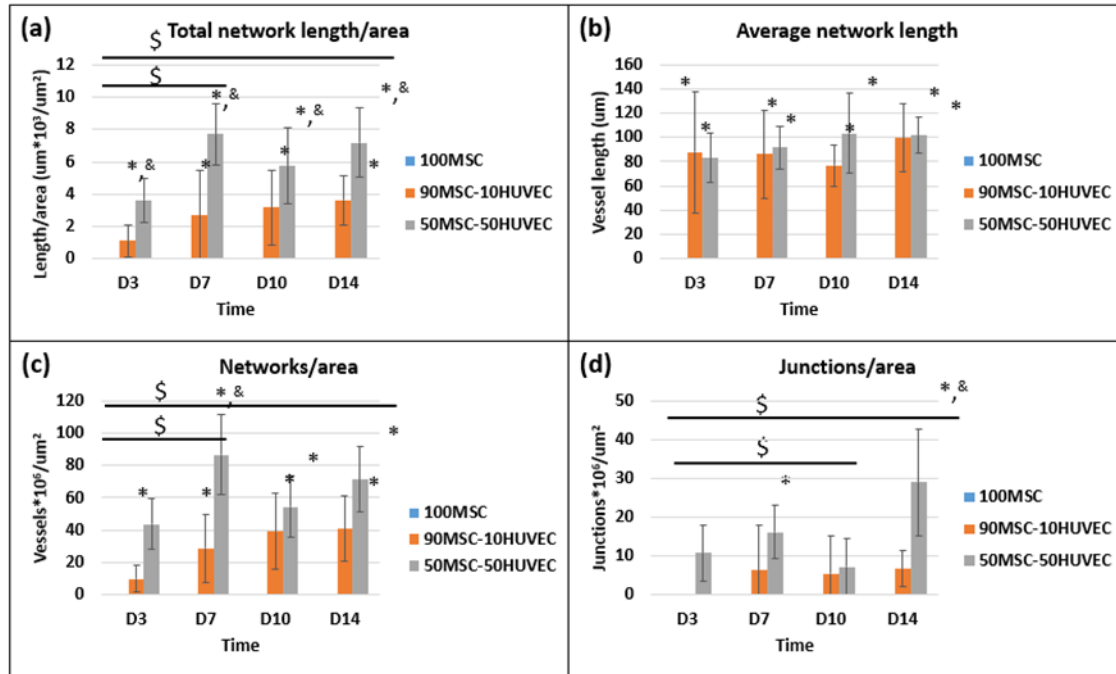


Figure 3. Quantification of prevascular structures in co-culture aggregates of 50,000 cells with MSC:HUVEC percentage compositions of 100:0, 90:10 and 50:50 at 3, 7, 10 and 14 days. (a) Total network length/area, (b) average network length, (c) networks/area, (d) and junctions/area were quantified via a blinded scoring system at days 3, 7, 10 and 14 of culture. Groups connected by a black horizontal bar are significantly different via one-way ANOVA at $p = 0.05$.

3.3 Evaluation of the effect of exogenous growth factor delivery on prevascular structure formation

To evaluate the role of known vasculogenic bioactive factors, VEGF and FGF-2, on prevascular structure formation, aggregates were cultured in EBM-2 supplemented with various concentrations of growth factors and compared to control groups cultured in EBM-2 or EGM-2. Prevascular structure formation was again evaluated by immunohistochemical staining for CD31 and laminin.

All media compositions led to at least some degree of assembly of endothelial cells into prevascular structures at both time points (Fig. 4a). All groups exhibited positive staining for laminin (Fig. 4b). Furthermore, all groups displayed some of the laminin to be cord-like, suggesting early basement membrane formation. Some regions of diffuse, non-linear laminin staining were also observed, particularly in samples cultured in EBM-2 and EGM-2. While there was a trend towards greater total network length per area at 7 days compared to 14 days (Fig. 5a) with several groups significant, this was reversed for the EGM-2 group. At day 7, the 100 ng/mL FGF-2 group total network length per area was significantly greater than the EGM-2 group (Fig. 5a). Neither media composition nor time was shown to significantly affect the average network

length in between most groups in this experiment (Fig. 5b). Time was shown to significantly affect the density of networks in the FGF-2 groups (Fig. 5c), with greater values at 7 days compared to 14 days. Junctions per area had high variability, resulting in no significant differences between groups (Fig. 5d). Like the total network length per area, there was again a trend towards higher values at day 7 compared to day 14. Similar results were obtained using a second HUVEC donor and are reported in the Supplementary File (Fig. S1).

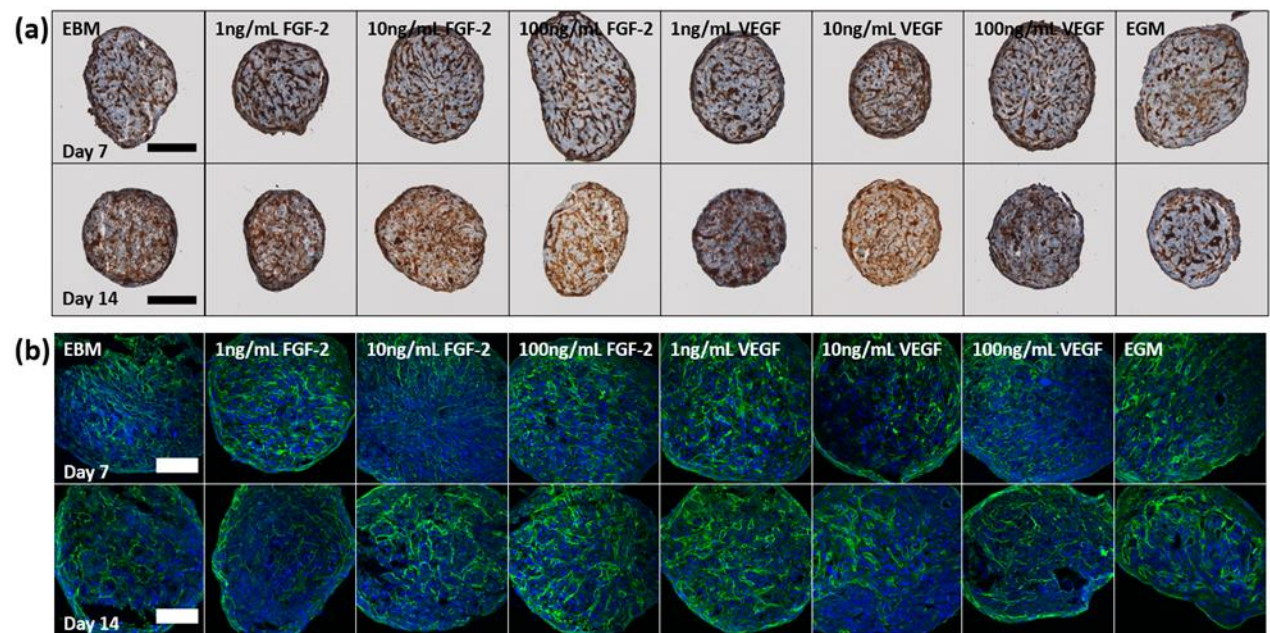


Figure 4. Prevascular structure formation in co-culture aggregates cultured in various media compositions. (a) CD31 and (b) laminin staining demonstrate the formation of prevascular structures and prevascular maturation respectively at 7 and 14 days of culture in either EBM supplemented with different levels of VEGF or FGF-2 or fully supplemented EGM-2. Black scale bars are 200 μm and white scale bars are 100 μm.

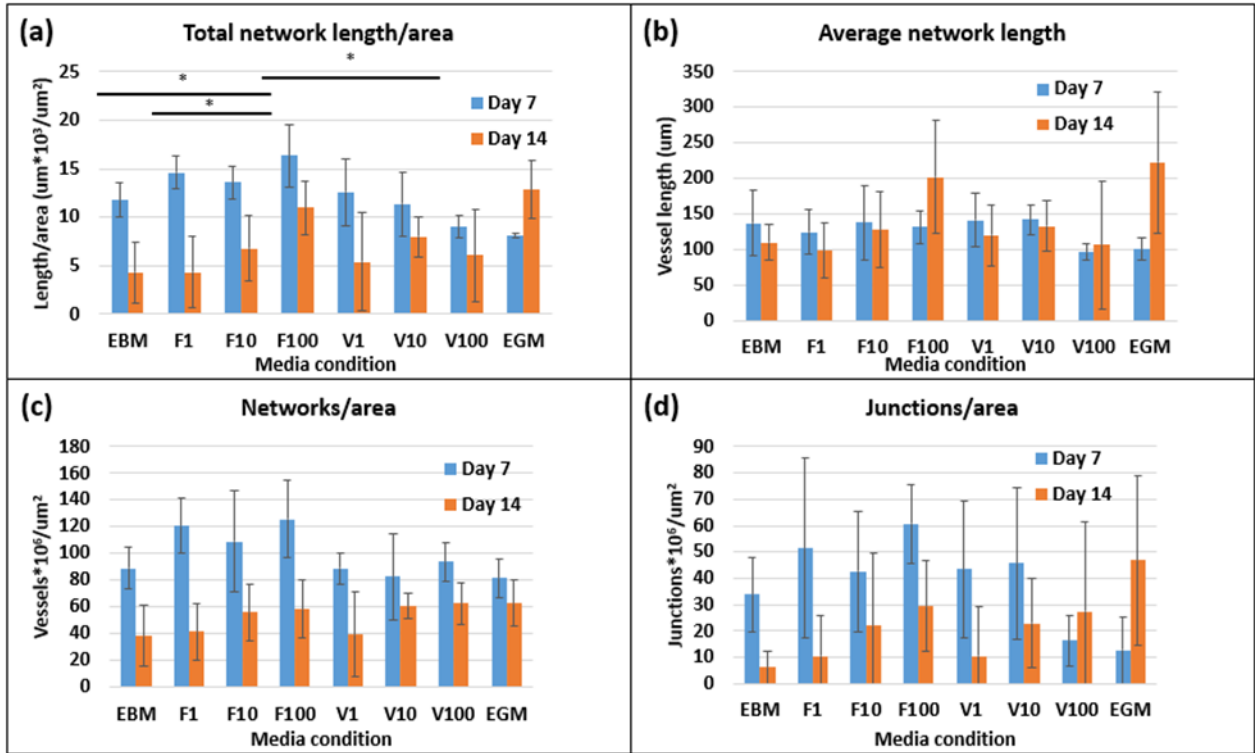


Figure 5. Quantification of prevascular structures in co-culture aggregates cultured in various media compositions. (a) Total network length/area, (b) average network length, (c) networks/area, and (d) junctions/area were quantified via a blinded scoring system at days 7 and 14 of culture in media containing either VEGF or FGF-2 individual supplementation or fully supplemented EGM-2. Groups connected by a black horizontal bar are significantly different via one-way ANOVA at $p = 0.05$.

3.4 Characterization of the morphology and size of alginate microgels

Safranin O staining is able to stain polysaccharides such as alginate and was used to stain alginate microgels to allow for identification under light microscopy. This showed the alginate microgels to have a range of sizes and irregular shapes (Fig. 6a). The diameter of the irregularly shaped microgels was taken to be the average of the longest and shortest diameter through the middle of the microgels. Measurement of 313 aggregates yielded a mean diameter and standard deviation of $23.3 \pm 11.2 \mu\text{m}$ and the distribution of sizes is displayed in a histogram (Fig. 6b).

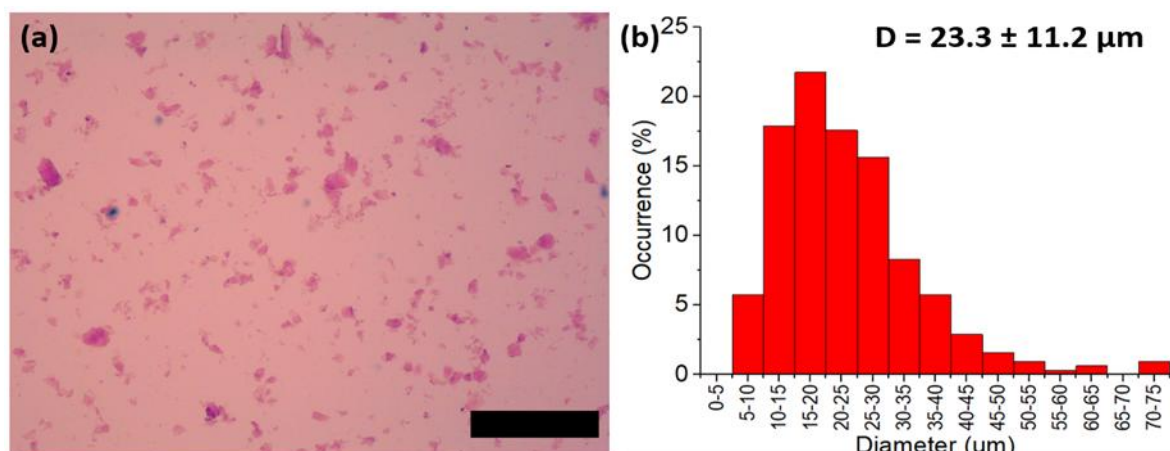


Figure 6. Alginate microgel morphology and size. (a) Alginate microgels were stained with Safranin O and imaged via light microscopy demonstrating the particles' morphology. (b) A histogram presents the relative frequency of diameters of 313 individual particles. Scale bar in (a) is 500 μm . D is average diameter of the microgels $\pm$ standard deviation.

3.5 Measurement of rheological properties of the alginate microgel slurry

Rheological properties of the slurry materials, including shear thinning and self-healing, are crucial for successful printing of cell aggregates within it. As shown in Fig. 7a, the alginate microgel slurry demonstrated shear thinning behavior, characterized by the decrease in viscosity with increasing shear rate. The alginate microgel slurry also exhibited low shear yield stress (Fig. 7b, $2.76 \pm 0.55 \text{ Pa}$), which was determined using shear stress sweep test. At approximately 15 % shear strain, the storage modulus (G') dropped below the loss modulus (G'') in oscillatory strain sweep test, thus indicating more liquid like behavior at higher shear strains (Fig. 7c). Frequency sweep test of the alginate microgel slurry from 0.1 to 10 Hz showed significantly higher G' than G'' , indicating that it was mechanically stable (Fig. 7d). Finally, upon cyclic strain sweep by alternating between low (1%) and high (100%) shear strains, the alginate slurry showed a reversible crossing over of G' and G'' (Fig. 7e), indicating the rapid self-healing ability of the material.

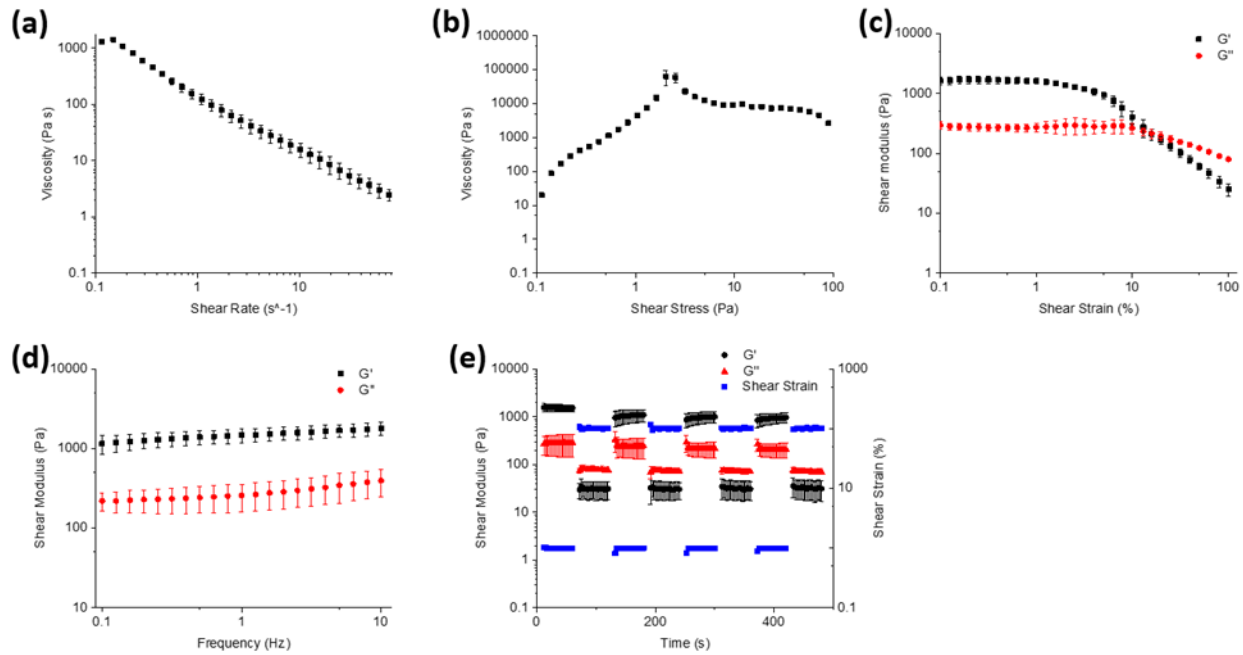


Figure 7. Rheological properties of alginate microgel slurry. Viscosity as a function of (a) shear rate and (b) shear stress. Shear moduli (storage, G' , and loss, G'') as a function of (c) shear strain and (d) frequency. (e) Shear moduli (storage, G' , and loss, G'') as the shear strain was periodically cycled over time between low (1%) and high (100%) values.

3.6 Measurement of rheological properties of the bioinks

The rheological properties of the bioinks with (Fig. 8) and without (Fig. 9) aggregates were measured. In all cases, the viscosity and storage and loss moduli were much lower than for the slurry material. The alginate-fibrinogen solution's viscosity increased above that of fibrinogen both in the presence (Fig. 8a) and absence (Fig. 9a) of aggregates with increasing shear rate with the overall effect of bioink composition reaching statistical significance in the absence of aggregates independent of shear rate ($p = 0.04$, Fig 9a). A similar trend was observed for viscosity of the two inks with respect to shear stress magnitude (Fig. 8b and 9b). However, due to the extremely low values, the error bars overlapped for many samples. Interestingly the G' was greater than the G'' for both inks with and without aggregates across most shear strains (Fig. 8c, 9c) and frequencies (Fig. 8d, 9d). G' , but not G'' , was statistically significantly higher for fibrinogen with cell aggregates compared to alginate-fibrinogen bioinks with cell aggregates across both shear strain and frequency ($p = 0.03$), while there was only a non-statistically significant trend towards a difference in G'' .

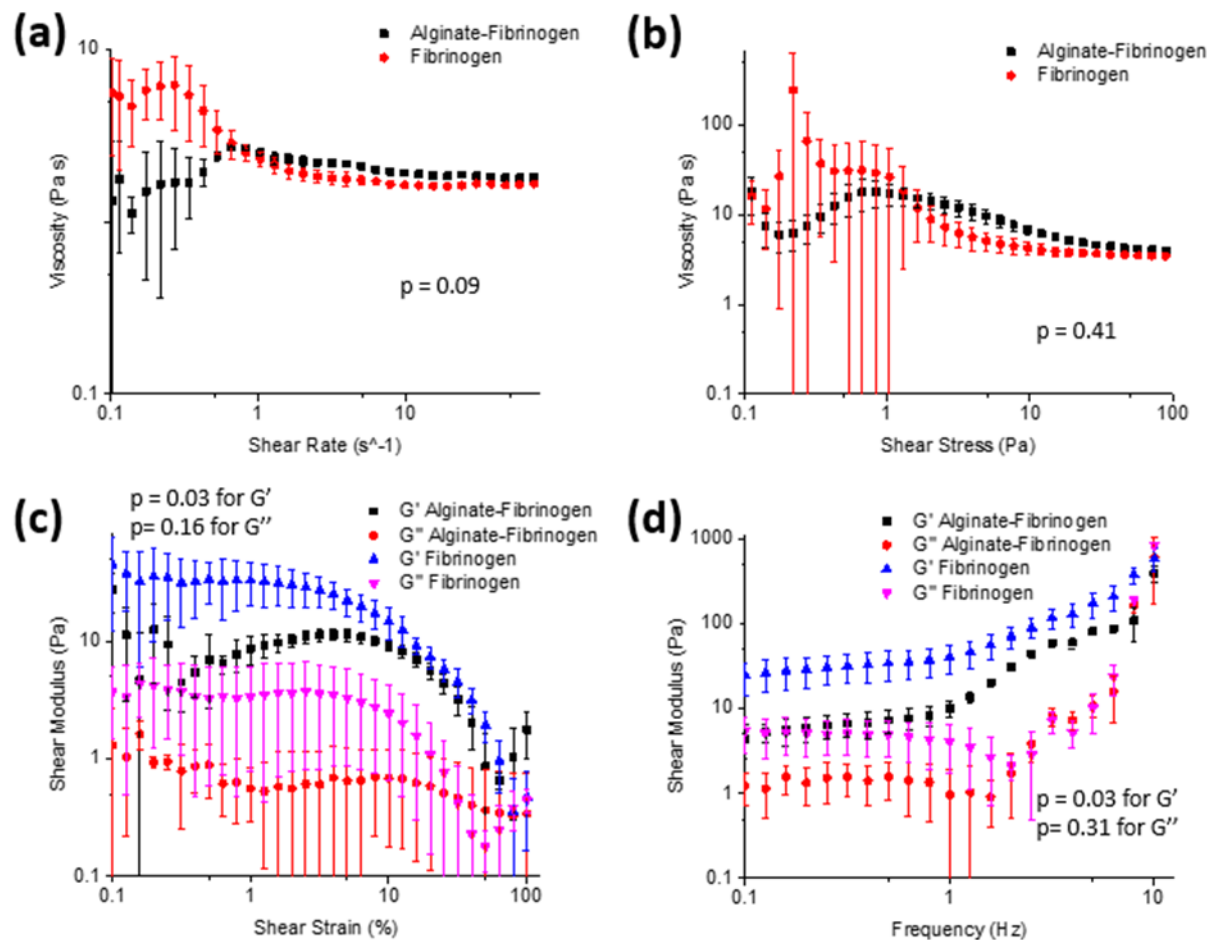


Figure 8. Rheological properties of the bioink with cell aggregates. Viscosity of the bioinks as a function of (a) shear rate and (b) shear stress. Shear moduli (G' , storage, and G'' , loss) of the bioinks as a function of (c) shear strain and (d) frequency.

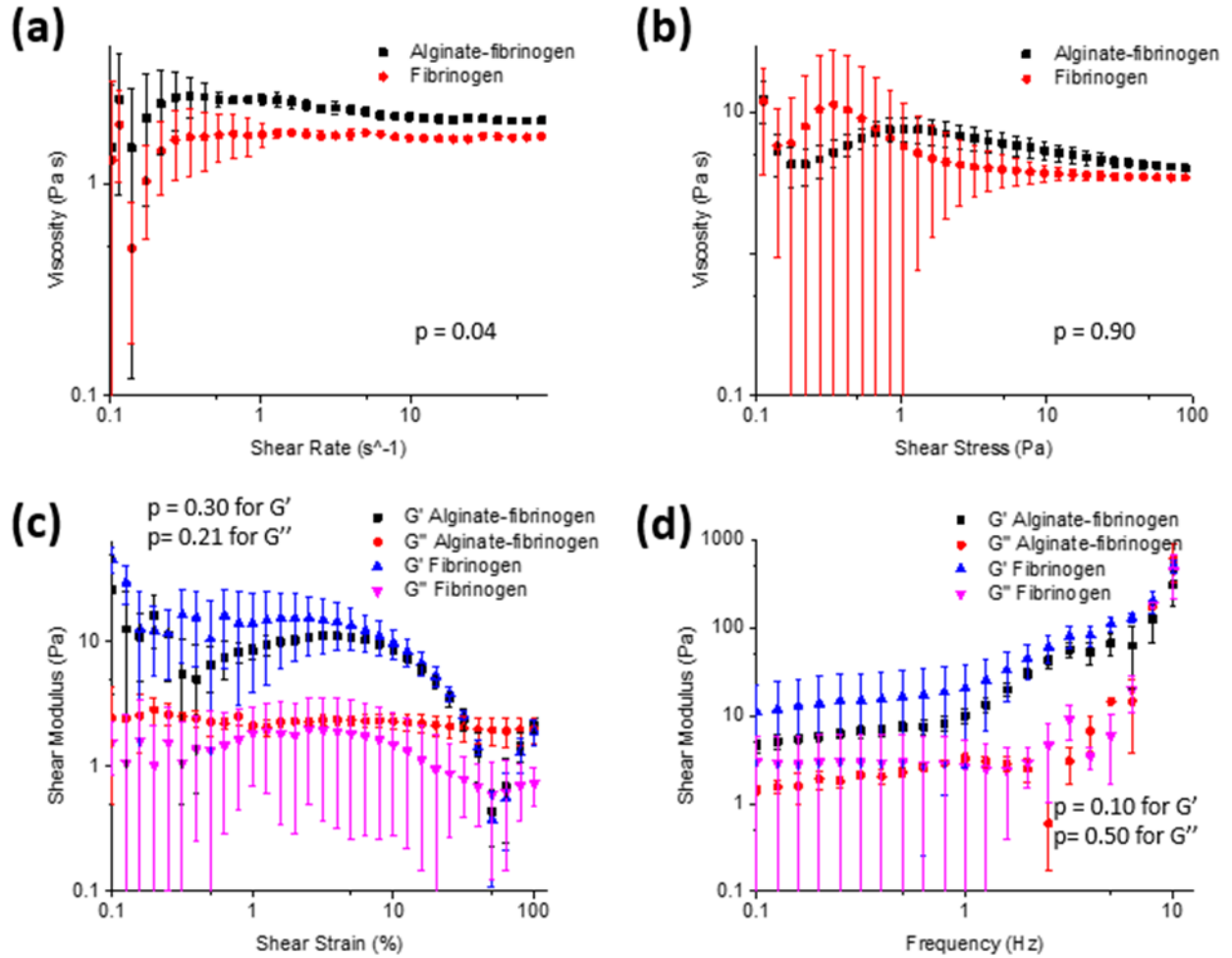


Figure 9. Rheological properties of the bioink without cell aggregates. Viscosity of the bioinks as a function of (a) shear rate and (b) shear stress. Shear moduli (G' , storage, and G'' , loss) of the bioinks as a function of (c) shear strain and (d) frequency.

3.7 Effect of different bioinks on the ability to 3D print prevascular aggregates

Prevascular aggregates were prepared as described above and cultured in either EGM-2 or EBM-2 with 100 ng/mL FGF-2. Both groups demonstrated robust prevascular structure formation in the aggregate only controls at 7 and 14 days as seen by CD31 staining (day 7 EGM-2 in Fig. 10a; day 7 FGF-2 in Fig. 10b; day 14 EGM-2 in Fig. 10c; and day 14 FGF-2 Fig. 10d). Printing the aggregates that were cultured in EGM-2 for 7 days in either fibrinogen only bioink (Fig. 10e) or alginate-fibrinogen bioink (Fig. 10g) and culturing for 7 additional days in the gel resulted in aggregates that maintained their prevascular structures. Conversely, the printing of aggregates that were cultured in EBM-2 with 100 ng/mL FGF-2 for 7 days in either fibrinogen only bioink (Fig. 10f) or alginate-fibrinogen bioink (Fig. 10h) and culturing for 7 additional days in the gel did not result in the maintenance of the prevascular structures that were seen in the day 7 control (Fig.

10b) or that were still robustly present in the day 14 control (Fig. 10d). It should be noted that upon drawing up the aggregate-bioink suspension into the printing syringe, when the bioink was fibrinogen only, the aggregates quickly settled to the bottom of the syringe before it could even be loaded onto the printer (Fig. 10i). Thus, only the first couple samples contained any aggregates in them. Conversely, the alginate-fibrinogen solution was more viscous and aggregates did not sediment out of the suspension during the time course of 3D printing (Fig. 10j). The printed constructs were sufficiently gelled, via thrombin crosslinking, at 1 day to be easily removed from the slurry with a spatula (Fig. 10k). The slurry was then completely aspirated off at the first complete media change 2 days after printing (Fig. 10l).

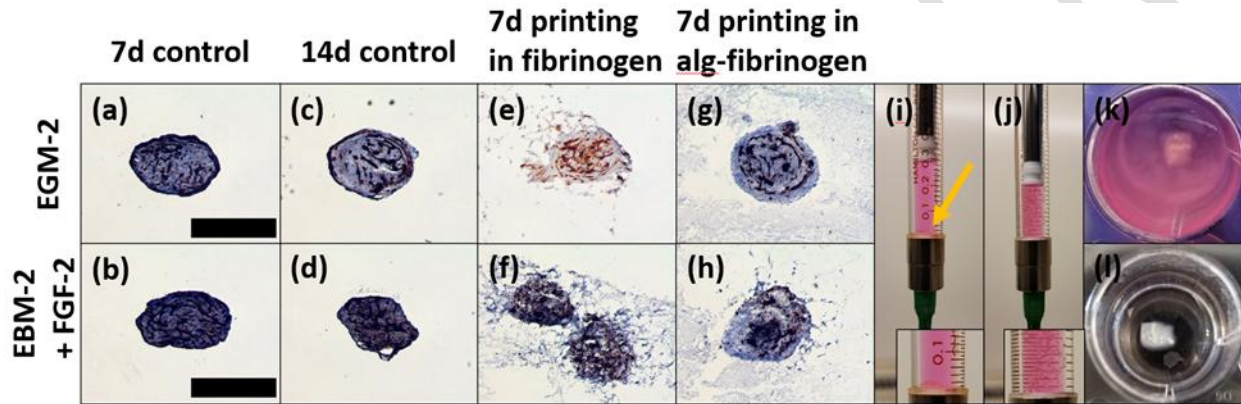


Figure 10. Gross and microscopic images of 3D printed and cultured prevascular aggregates. Non-printed controls at day 7 cultured in (a) EGM-2 or (b) EBM-2 with 100ng/mL FGF-2 and day 14 (c and d, respectively) showed prevascular structure formation via CD31 staining. Prevascular structures were also observed following 7 days of culture in EGM-2 after 3D printing aggregates in either (e) fibrinogen or (g) alginate-fibrinogen bioink. However, vascular structures were not observed after 7 days of culture in EBM-2 with 100 ng/mL FGF-2 in aggregates printed in either (f) fibrinogen or (h) alginate-fibrinogen ink. Aggregates settled quickly in (i) fibrinogen ink but not (j) alginate-fibrinogen ink. A printed 3x3x3 mm cube can be seen (k) immediately after being released from the slurry and (l) after transfer of the construct to PBS. Scale bar is 200 μ m.

3.8 3D printing prevascular aggregates into constructs of different geometries with varied needle sizes.

Prevascular aggregates were again cultured as described above in EGM-2 and printed at 7 days at 10 aggregates / 27 μ L of bioink to form constructs of different geometries. Controls harvested at day 7 (Fig. 11a) and 14 (Fig. 11b) demonstrated the presence of prevascular structures throughout the duration of the experiment. Prevascular aggregates were shown to be able to be printed in a large (8 mm diameter) 'C' shape (Fig. 11c) using an 18G needle and maintain their prevascular structures for 7 days in culture (Fig. 11e). The individual aggregates can be seen in a gross image of the printed construct as tan dots on an opaque white gel in the shape of a 'C' (Fig. 11c). Aggregates were then printed using a smaller, 20G needle, this time again in the shape of a

cube (Fig. 11d). Even when printed through a much narrower orifice, the prevascular structures survived and were maintained throughout a week of culture (Fig. 11f).

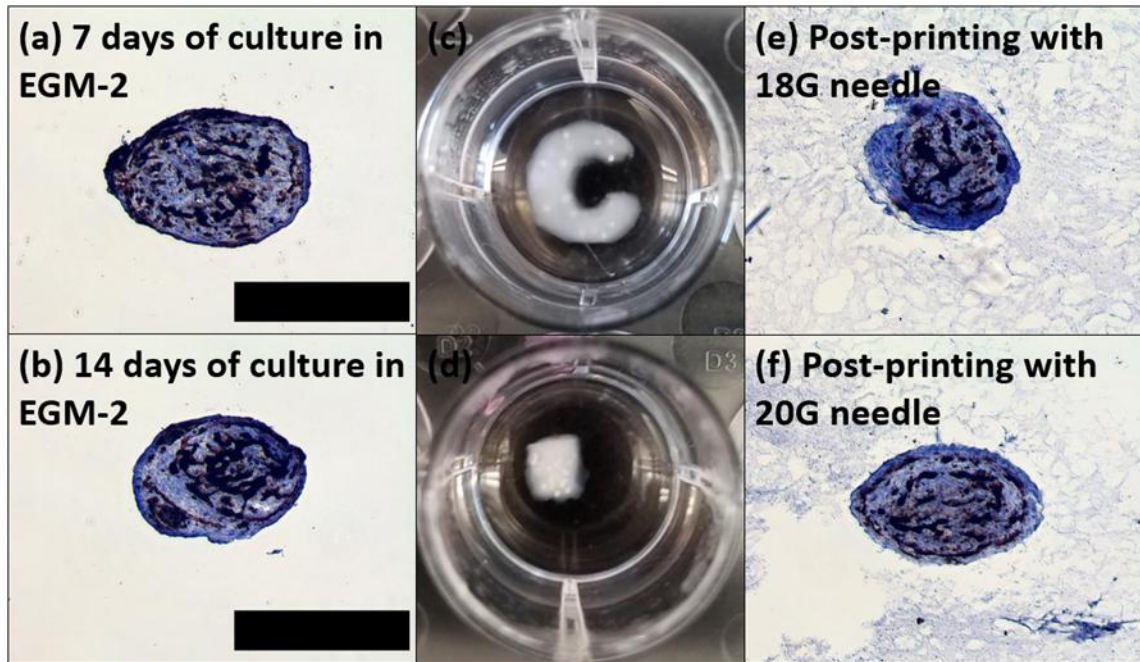


Figure 11. Aggregates printed in different shaped constructs and with different sized needles. Aggregates cultured in EGM-2 for (a) 7 or (b) 14 days demonstrated prevascular structure formation. This 3D printing technique was shown to be able to print multiple shapes including (c) the letter ‘C’ and (d) a cube. The aggregates maintained their prevascular structures for 7 days after being printed with either (e) 18G or (f) 20G needles.

3.9 3D printing aggregates prior to prevascular structure formation

Aggregates were printed 2 days after formation, which is prior to self-assembly of prevascular structures within the aggregate (Fig. 12a), to determine if pre-assembly of the structures is required or if they can assemble within the printed constructs. Control aggregates, which were not printed, and printed samples, which were printed 2 days after formation, were cultured in either EGM-2 or EBM-2 plus 100 ng/mL FGF-2 after day 2 and were harvested at 7 and 14 days. Controls demonstrated prevascular structure formation by both 7 and 14 days (Fig. 12b-c and Fig. 12f-g respectively) when grown in either EGM-2 (Fig. 12b,f) or FGF-2 (Fig. 12c,g), but not by 2 days (Fig. 12a). Only limited prevascular structures were seen in aggregates that were printed 2 days post-formation and harvested at either 7 or 14 days post-formation (Fig. 12d-e and Fig. 12h-i, respectively) when grown in either EGM-2 (Fig. 12d,h) or FGF-2 (Fig. 12e,i).

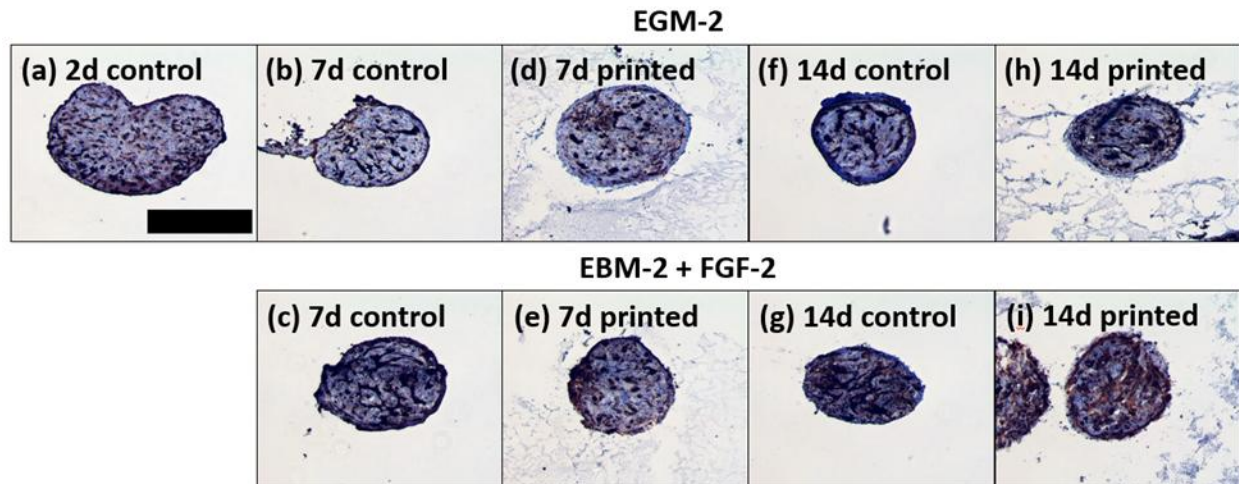


Figure 12. Aggregates 3D printed at 2 days, before self-assembly of prevascular structures. Controls cultured in (a, b, f) EGM-2 or (c, g) EBM-2 with 100 ng/mL FGF-2 were harvested at (a) 2, (b, c) 7 and (f, g) 14 days after being. Aggregates printed 2 days after formation were cultured in either (d, h) EGM-2 or (e, i) EBM-2 with 100 ng/mL FGF-2 and harvested on (d, e) day 7 or (h, i) 14. Scale bar is 200 μ m.

4. Discussion

This high cell density, co-culture aggregate system has been shown to result in significant prevascular structure formation. In the current study, aggregates did not form well when the composition was predominantly endothelial cells (i.e., hMSCs:HUVECs ratio of 1:9 or lower). Such cellular compositions resulted in numerous small cell clumps within the well. However, most cells were incorporated within a single main aggregate at cell ratios of 1:1 hMSCs:HUVECs and higher. Published results of endothelial cell only aggregates are typically of a much smaller size and are formed in hanging drop systems.²³ While these hanging drop systems allow for the forced incorporation of all cells into the aggregate, they limit total aggregate size and the ability to culture the aggregates in vitro for an extended period of time. The current system allows for the formation of different sized aggregates with adequately high hMSCs:HUVECs cell ratios as well as the culture of these aggregates for multiple changes of different media types in vitro. This allows for the interrogation of the effects aggregate cellular composition and media composition on prevascular structure formation, which in initial studies with this system has been shown to have an ability to integrate with host vasculature and result in perfusion of a tissue engineered construct.²⁴ This study showed that it was specifically the transplanted HUVECs that were integrated into perfused vessels. Similar in vivo studies demonstrating the contribution of transplanted endothelial cells in 3D bioprinted constructs to perfused vasculature will be crucial next steps in the development of the present technology.

The high cell density aggregate system may recapitulate some of the early developmental microenvironment of the mesoderm that results in the development of the circulatory system. The mesenchymal condensations of the embryonic mesoderm result in blood island formation, from which the blood vessel cells (i.e., endothelial cells, smooth muscle cells and pericytes), are derived.

Thus, a cell aggregate system that incorporates both of these cell types may be able to recapitulate this embryonic process and mimic natural prevascular cord and plexus formation in the blood islands. The cells used in the present study were adult bone marrow derived MSCs, which may function as pericytes,²⁵ and HUVECs, forming the inner lining of the vessels. Both cells are more mature than the cells resulting from the differentiation of hemangioblasts in blood islands. However, with the growing understanding of the role of endothelial progenitor cells (EPCs), it is now well established that not just angiogenesis, but also vasculogenesis does occur in the adult in certain tissue environments.²⁶ Indeed, previous studies of human endothelial cells and fibroblasts have demonstrated early prevascular structure formation^{27,28}, as have studies using a mix of human endothelial cells and mouse mesenchymal cells.^{13,29,30} In addition to their xenogeneic sourcing, these aggregates were cultured on Matrigel, limiting the interpretation of the clinical translatability. However, they were shown to enhance vascularization in a mice hind limb ischemia model.³⁰

In the present study, aggregates comprised of 50,000 up to 500,000 cells were evaluated (Fig. 1). Aggregates greater than 100,000 cells did not form well consolidated aggregates, with some cells forming separate smaller aggregates in the well. Thus, there may be diminishing marginal utility of delivering additional cells beyond 100,000. Furthermore, given that aggregates formed from the two larger cell numbers had diameters greater than the inner diameter of an 18G printing needle, it was determined that further studies should proceed with the smallest aggregate size possible, such that prevascular structures still formed, to facilitate future 3D printing experiments. The 50,000 cell aggregates, with a mean diameter of 537 μm at 7 days fulfill this criterion. Furthermore, for diameter the center of the aggregates is slightly over 250 μm from the surface, which is just beyond the diffusional limitation of oxygen in tissue and therefore should be adequate to provide a pro-vasculogenic stimulus to the aggregates. Prevascularized aggregates printed by another group were reported to have diameters in the range of 150-200 μm ,^{21,31} considerably below the diffusional limitation of oxygen. It should be noted that because histological sections rarely can be taken exactly from the middle of these aggregates, the apparent diameter of histological sections typically appeared smaller than that reported from measurements of the whole mount aggregates (Fig. 1i).

The present study also demonstrated a minimum of 7 days for prevascular structure formation in EGM-2, a media that contains many supplements including the growth factors, VEGF, hFGF-2, insulin-like growth factor 1 (IGF-1) and human epidermal growth factor (hEGF). Further prevascular structure development was seen out to 14 days. Previous reports have shown prevascular structure formation of cell ratios of 92:8 MSCs to HUVECs as well as 50:50 MSCs to HUVECs.^{30,32} A similar ratio with predominantly MSCs (90:10 MSCs:HUVECs) used in this study show that this system can also allow for prevascular structure formation with a low fraction of HUVECs, however, greater prevascular structure density was seen at the 50:50 cell ratio and thus this ratio was used for subsequent experiments. No vascular structures were seen in aggregates composed entirely of MSCs. Interestingly, laminin deposition was seen in all groups. This indicates that the MSCs in isolation contribute significantly to laminin deposition when exposed to the pro-vasculogenic concoction of growth factors in EGM-2. The most prominent prevascular

structures were seen in the 50:50 cell ratio group and notably much of the laminin staining was in a cord- or plexus-like pattern. Given the positive staining of nuclei within most of these round, vessel-like structures, these are not actually mature lumenized vessels yet. However, the linear histologic arrangement of laminin, compared to other reports demonstrating only diffuse deposition of laminin^{30,33}, represents a more physiological framework for vascular network formation.

Another interesting finding is the formation of prevascular structures in all media compositions. This is perhaps not so surprising as there is a large literature describing the crosstalk between endothelial cells and stromal supporting cells, whether that be smooth muscle cells, pericytes, MSCs, fibroblasts, osteoblasts, etc.³⁴ Reciprocal signaling involving VEGF and PDGF-BB are known to be involved in endothelial cell-MSC interactions and likely contribute to the formation of vascular structures as well as the stabilization of those structures to prevent regression.³⁵ Interestingly, while total network length per area, total networks per area and junctions per area were all significantly higher at 7 days than at 14 days in all non-EGM-2 groups, this trend was reversed in the EGM-2 group (Fig. 5). This may indicate an enhanced stabilization of the prevascular structures due to some component of the media. Alternatively, it may also simply represent a slower vasculogenic process. Remodeling is known to play a major role in neovascularogenic processes.³⁶ It is likely playing some role here, as some degree of prevascular maturation is occurring as basement membrane deposition occurs as evidenced by the positive staining for laminin. It should be noted that, per the manufacturer, the media composition of EGM-2 was optimized for endothelial cell culture and expansion, not necessarily for vasculogenesis. Individual growth factor supplementation may be better able to drive the vasculogenic phenotype as opposed to the proliferative phenotype seen in 2D with culture in EGM-2.

The particular growth factor supplementation applied significantly affected the total network length per area. Treatment with 100 ng/mL FGF-2 resulted in significantly more network length/area than treatment with fully supplemented EGM-2 at 7 days, and the highest value of any group tested (Fig. 5). Prevascular structures were also formed in a focused study using a second HUVEC donor (see Fig. S1), supporting the generalizability of these findings. This suggests that treatments with high levels of FGF-2 may best be able to drive early vasculogenesis. This is consistent with FGF-2's role in embryogenesis.³⁷ While VEGF has been widely investigated in the therapeutic neovascularization literature, it is most widely known for its role in angiogenesis.³⁸ Since angiogenesis is the development of new blood microvasculature from preexisting microvasculature made of mature endothelial cells, it may be that FGF-2 plays a bigger role in the de novo development of microvasculature in the current high cell density co-culture system. This is, however, complicated by the fact that more mature cells were used in the present study compared to the hemangioblasts that are responsible for embryonic vasculogenesis.

Much like the group treated only with EGM-2, all groups treated with other media compositions demonstrated the deposition of laminin (Fig. 4). Linear structures were seen in most groups and were most prominent at higher concentrations of FGF-2. In particular, and in contrast to prevascular structure formation as evidenced by CD31 staining, prominent linear laminin

structures were more abundant at 14 days than at 7 days. This is likely since laminin is a marker of more mature vasculature as it is a major component of the basement membrane, upon which all endothelial cells in mature vasculature sit.³⁹

A novel slurry bath was then utilized that we engineered to allow for the 3D printing of cell and aggregate suspensions in macromer solution bioinks as well as macromer solution-free cell-only and cell aggregate printing.^{20,40} The only previous reports to date of 3D printing prevascularized aggregates utilized aggregates sized well within the limitation of oxygen diffusion and after in vitro culture only simple vascular networks resulted^{21,31} or the prevascular structures did not survive printing.^{41,42} Here, the present work demonstrates a system that allows for the 3D printing and subsequent culture of prevascularized aggregates with a radius greater than the diffusion limitation of oxygen and with a robust microvasculature that is stable through the 3D bioprinting process.

One key limitation to many 3D bioprinting approaches is that the bioinks are relatively weak and slow to gel. Therefore, recent work has developed 3D printing technologies that allow for the 3D printing of cell-laden macromer solutions and/or cell-only bioinks into microgel support slurries, followed by subsequent gelation of the ink or self-assembly of the cells into a stable construct with either early or late removal of the supporting slurry bath. Here a slurry of alginate microgels was developed to serve this role. Unlike a gelatin microgel-based slurry,¹⁷ the present ionically-crosslinked alginate microgels in the slurry don't degrade quickly upon culture at 37 °C (due to the slow rate of calcium leaching out⁴³), but rather can be dual-crosslinked with light to permit enhanced stabilization of the print and then hydrolytically degrade or gently removed mechanically. In the current system, fibrinogen bioinks were used along with thrombin containing slurries. The stability of the current slurry allowed for maintenance of the printed hydrogel structure over the course of 1 day at which point enough thrombin had diffused into the printed construct and polymerized the fibrinogen into a fibrin gel.

Another critical characteristic of the slurry is its rheological behavior.^{17,19} Rheological testing (Fig. 7) of the slurry showed classic shear thinning behavior with a decrease in viscosity with increasing shear rate. That, combined with the yield shear stress occurring at a sufficiently low value, allows for easy deformation by a 3D printing needle and bioink extrusion, a key feature of slurry-based 3D bioprinting to allow for the formation of complex structures. The shear yield stress of the slurry (2.76 Pa) is below the stresses typically experienced by cells during the printing process itself (often times several thousand Pa),⁴⁴ thus enhancing cytocompatibility of this bioprinting process within the slurry. Shear strain and frequency sweeps demonstrate a transition from predominantly solid-like behavior to predominantly liquid-like behavior at shear strains above approximately 10% and the relative stability of the slurry to variations in frequency of shear, respectively. These combined with the cyclic recovery of elastic and viscous moduli when subjected to alternately low and high shear strain demonstrate the ability of the slurry to act like a liquid when the printing needle is passing through it to allow for the printing of geometrically precise constructs, but maintain a relatively solid like behavior when the shear of the needle is removed, thus permitting stabilization of the printed construct while it gels. Taken together, these

properties indicate that the slurry material behaves in an appropriate manner for a hydrogel suspension type 3D printing approach.¹⁷ The properties of the alginate slurry made it an ideal initial candidate for the present 3D bioprinting application.

A commonly used biomaterial for vascularization studies is fibrin. This natural material is involved in the first step of wound healing and therefore is an obviously natural choice for evaluation in vasculogenic processes as those too are involved in wound healing. When aggregates are suspended in a 5 mg/mL fibrinogen solution (Fig. 10i) or a 10 mg/mL fibrinogen solution (data not shown), the aggregates quickly settle to the bottom of the syringe before the printing process can even begin. Thus, the first several constructs to be printed will have many aggregates in them, but later printed constructs will lack aggregates. This does not allow for production of printed constructs with a relatively consistent number of aggregates per construct. Therefore, an attempt was made to increase the viscosity of the printing bioink such that the sedimentation of aggregates in the bioink would be negligible or non-existent during the timeframe of the printing process. Native alginate can have a relatively high viscosity at low concentrations, and it was therefore hypothesized that mixing an alginate solution with the fibrinogen solution might result in a sufficiently viscous bioink such that aggregates would not sediment out during the timeframe of the printing process, but that would still allow for the polymerization of the fibrinogen component of the ink and the successful maintenance of prevascular structures in the embedded printed aggregates.⁴⁵ Unlike fibrin, human cells are not able to produce enzymes that digest alginate.⁴⁶ Due in large part to its mostly bioinert nature, alginate was chosen as a bioink thickener. Rheological analysis of the bioink with alginate showed higher viscosity as a function of shear rate than for bioink without alginate (Fig. 9) and a similar trend was seen for the bioink with aggregates, although significance was not reached ($p = 0.09$, Fig 8). When aggregates were suspended in this bioink composed of fibrinogen and alginate, they stayed suspended for the duration of the 3D printing process (Fig. 10j).

Prevascular aggregates for 3D printing were cultured in either EGM-2 or EBM-2 plus 100 ng/mL FGF-2 (called the FGF-2 group). EGM-2 was selected as one candidate culture medium as it is widely used for the culture of endothelial cells and for vascularization studies. Based on the results above, 100 ng/mL FGF-2 was selected as a comparison group due to the demonstrated robust prevascularization shown in aggregates treated with those conditions. Aggregates cultured in EGM-2 were shown to maintain their prevascular structures after printing at 7 days and a further 7 days of culture in the printed constructs when printed in either alginate-fibrinogen (Fig. 10e) or fibrinogen only bioink (Fig. 10g). However, paradoxically, FGF-2 group aggregates did not maintain their prevascular structures throughout the 7 day culture in the printed constructs in either alginate-fibrinogen (Fig. 10f) or fibrinogen only bioink (Fig. 10h). This surprising result is in spite of the fact that the unprinted controls continued to show robust prevascular structures (Fig. 10d). FGF-2 is known to bind both fibrinogen and fibrin, and this may lead to sequestration of this growth factor.⁴⁷ It is possible that in the FGF-2 only group, if the growth factor is sequestered, the incorporated aggregates may not be exposed to high enough concentrations, leading to prevascular structure regression and potentially even cell death. Should greater FGF-2 mediated

vasculogenesis be desired in future studies, the impact of potential FGF-2 sequestration in the bioink could be further investigated. It may also be that the surrounding microenvironment in the printed constructs limited nutrient diffusion to the aggregates due to the surrounding alginate and/or fibrinogen gel. The aggregates cultured in the fully supplemented EGM-2 may have survived better due to a marginal benefit of the additional growth factors and serum in that medium that was not necessarily the case when cultured in the less restrictive environment outside of a printed hydrogel. While it is questionable whether traditional live/dead dyes would reliably penetrate these high cell density aggregates, the maintenance of vascular structures for days after printing is taken as evidence of limited apoptosis being induced by the printing process itself. Interestingly, this same disparity in prevascular structure maintenance between EGM-2 and FGF-2 groups was seen in both the alginate-fibrinogen co-bioink as well as the fibrinogen-only bioink, suggesting that it was not simply the addition of the alginate to the gel matrix that pushed the aggregates past a stress point beyond which they could not survive. It is likely that the prevascular structures in the aggregates described herein were better maintained than in previous reports³¹ due to the fact that the aggregates were much larger and thus had a more hypoxic core from the beginning of culture, which has been shown to result in enhanced HIF-1 α and VEGF expression as well as better functional outcomes in a hind limb ischemia model when compared to smaller aggregates.⁴⁸ Future printing work may evaluate multiple growth factor delivery either in the media or in co-embedded microparticles to support the embedded prevascular aggregates.

Subsequently, after a baseline system had been developed in which prevascular structures could be maintained successfully, printing parameters were varied to evaluate the versatility of the system. Based on the results in Fig. 10, all aggregates were cultured in EGM-2 for these experiments and demonstrated prevascular structures in control samples at both the time of printing (Fig. 11a) as well as the time of harvest (Fig. 11b) of the printed constructs. In the first study, the letter 'C' was successfully printed with embedded aggregates and the fibrinogen solution polymerized quickly enough in the thrombin containing slurry to allow for stabilization of the entire construct and release for the alginate microgel slurry at day one (Fig. 11c). Prevascular aggregates cultured in this larger, more complex structure compared the 3x3x3 mm cube, maintained their prevascular structures for the 7 day duration of culture post-printing (Fig. 11e). Thus, even in larger constructs, the prevascularization strategy may be successful. Ultimately, there exists some upper threshold of size beyond which no cells or prevascular structures will survive. To print truly large tissue constructs of clinically translatable size, the aggregates will be able to facilitate rapid formation of a microvasculature, but some type of bioreactor based macrovascular perfusion system will have to be rapidly adopted to take advantage of the prevascularization provided by the aggregates in the current system. Furthermore, some tissue engineering applications will require precise spatial arrangement of the tissue. Printing precision is significantly limited by the size of the printing needle. However, there is a tradeoff: the needle should be small enough to allow for the printing precision that the job calls for, but the aggregates must be large enough in diameter to allow for enough hypoxic stimulus in the interior to drive a self-assembly prevascularization process of the incorporated endothelial cells. The printing needle

must therefore be large enough to allow passage of the aggregate without lethally high shear stress levels. The experiments reported earlier in the work used an 18G needle with an 838 μm inner diameter. Given that the aggregates have a diameter on the order of half of that, the final study evaluated the ability of the aggregates to be printed from a 20G needle, with a 603 μm inner diameter, and subsequently cultured for 7 days. A 3x3x3 mm cube was again printed (Fig. 11d), but with a 20G needle. Encouragingly, the aggregates survived the printing process intact and demonstrated the presence of prevascular structures at harvest 7 days later (Fig. 11f). This suggests that aggregates of 50,000 cells may be printed with at least as small as a 20G needle, although for most routine applications not requiring exceptional precision, a larger needle may still be preferable to minimize shear stresses on the aggregates during the printing process.

Finally, it was demonstrated that aggregates printed at day 2, prior to prevascular structure formation (Fig. 12a), did not form as prominent of prevascular structures over the course of culture out to 7 (Fig. 12d-e, h-i) or 14 days regardless of media composition, as did unprinted control aggregates at both 7 (Fig. 12b, c) and 14 days (Fig. 12f, g) in both EGM-2 and EBM-2 (Fig. 12b, f) with 100 ng/mL FGF-2 (Fig. 12c, g). This finding supports the need to form prevascular structures prior to 3D printing as the printed environment may be too nutrient or signal limited to allow for adequately fast vascularization for survival of implanted constructs. This key finding highlights the importance of forming prevascular structures prior to printing and the ability to maintain those structures upon culture post-printing.

Cell sourcing will be a key consideration for clinical translation. Given that EPCs are found in the bone marrow at a low concentration,⁴⁹ some may have been isolated with the hMSCs and been included in cellular aggregates. The expansion of the hMSCs in culture prior to incorporation into cellular aggregates, potentially preferentially expanded hMSCs and/or led to the dedifferentiation of many EPCs since no specific vasculogenic factors were added to the growth medium. Given the superiority of the use of autologous cells, EPCs may be seen as a natural replacement for HUVECs in future studies. One type of EPCs, endothelial colony forming cells have been shown to be able to form co-culture aggregates of 600 cells with MSCs.⁵⁰ While no prevascular structures were shown within these aggregates, the cells were shown to migrate out of the aggregates and into a surrounding collagen gel in a vasculogenesis assay. In the future, it may be valuable to utilize EPCs or develop a system that allows immediate implantation of a stromal vascular fraction, which may still contain an appreciable number of endothelial cells.

Future studies will also need to evaluate higher aggregate density in alginate-fibrinogen bioinks. This will allow for closer aggregate contact within the printed constructs. Thus, the aggregates may be able to fuse into a single tissue construct and the bridging of prevascular structures between separate aggregates can be evaluated. This will likely play an important role in the construct's ability to anastomose with host vasculature upon eventual implantation in vivo.

5. Conclusion

The high-cell density system described herein was able to successfully recapitulate the early stages of embryonic vasculogenesis via the self-assembly of prevascular structures over the

course of one to two weeks. Use of defined media with single growth factor supplementation resulted in maturing vascular networks. To ultimately facilitate clinical translation, a crosslinkable, 3D bioprinting ink and temporary biomaterial slurry that allow printing of viable prevascular aggregates in various shapes have been developed and characterized. Future work may investigate the delivery of growth factors from within the printed gel itself, perhaps within co-embedded growth factor loaded microparticles. This system may find application in many tissue engineering applications due to its versatility. Aggregates of other tissue types such as pancreatic islet cells or hepatic sinusoid organoids may be co-printed with prevascular aggregates to generate vascularized functional parenchymal tissue constructs. Ultimately, while the current system may allow for rapid development of a microvasculature in such constructs, integration with a macrovasculature will ultimately be required for clinical success.

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Conflict of interest

Eben Alsberg is an Editorial Board Member of this journal, but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. The authors declare they have no competing interests.

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