ENDOTHELIAL PROGENITOR CELL RECRUITMENT IN A WOUND HEALING MICROFLUIDIC VASCULAR MODEL

by

Daniel Matthew Lewis

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Abstract

During wound healing, endothelial progenitor cells (EPCs) are recruited from the bone marrow and directed to the site of injury. At the site of injury, hypoxic conditions promote TNF-α, which up regulates intercellular adhesion molecule-1 (ICAM-1). EPCs use ICAM-1 to attach to endothelial cells (ECs) lining blood vessels. Here, we design, develop and test a three-dimensional microbioreactor system (3-D MBR) with precise control and monitoring of oxygen and media flow rate. We first analyze the transport of oxygen in the proposed device. Following fabrication of the 3-D MBR, we next utilized a step-wise seeding technique, which resulted in confluency of human umbilical vein endothelial cells (HUVECs) on all four sides of the device. We next examine endothelial colony forming cell (ECFC) attachment and retention onto HUVECs using conventional 2-D cultures. HUVECs are pre-stimulated with one of four conditions: 21% oxygen (atmospheric), atmospheric with TNF-α-supplemented media, 1% oxygen (hypoxia), and lastly hypoxia with TNF-α-supplemented media. We show the highest attachment and retention of ECFCs on HUVECs pre-treated with TNF-α and 1% oxygen, which correlated with the highest expression levels of ICAM-1. Using the new 3-D MBR system we next demonstrate that TNF-α and hypoxia, when used in conjunction, significantly increase EPC attachment on ECs under pathologically relevant flow conditions. The 3-D MBR system allows us to mimic the oxygen and shear stress environment in the vasculature, thus providing a step between traditional *in-vitro* and *in-vivo* experimentation to model a variety of vascular-related disorders, especially wound healing.

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List of Symbols and Abbreviations

2-D	Two-Dimensional
3-D	Three-Dimensional
3-D MBR	Three-Dimensional Microbioreactor
c	Concentration (M)
D	Diffusivity (cm^2/sec)
EC	Endothelial Cells
ECFCs	Endothelial Colony Forming Cells
EPC	Endothelial Progenitor Cells
Fn	Fibronectin
hc	Height of the Channel in the 3-D Microbioreactor (μm)
hm	Height of the PDMS wall above and bellow the channel (μm)
hmin	Height of the channel (cm)
HUVECs	Human Umbilical Vein Endothelial Cells
ICAM-1	Intercellular Adhesion Molecule-1
MBR	Microbioreactor
p	Pressure (atm)
PDMS	Polydimethlysiloxane
PMMA	Poly (methyl methacrylate)
Qmin	Flow Rate (mL/H)
R	Species Generation (mole/sec)
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction

TNF-alpha	Tumor Necrosis Factor Alpha
u	Velocity (m/s)
w	Width of Channel (cm)
μ	Dynamic Viscosity (poise)
ρ	Density (g/mL)
τmin	Shear Stress (dyne/cm^2)

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Introduction

Vasculogenesis is the growth of a primitive vascular network using endothelial cells (ECs) that have been differentiated from endothelial progenitor cells (EPCs)[1]. EPCs are immature endothelial cells originating from and stored in the bone marrow[2] with a high migratory nature[1]. EPCs are recruited from the bone marrow to the wound-healing site through paracrine signaling. At the wound site, damaged blood vessels limit blood flow and cause less oxygen to travel to the area of trauma (hypoxia)[3]. Hypoxia in the wound site can increase the demand for oxygen[4]. The hypoxic environment promotes an increased release of tumor necrosis factor- α (TNF-α), a pro-inflammatory cytokine that is secreted by injured vascular ECs. TNF- α signals inflammatory cells to migrate into the injury site in order to initiate the inflammatory phase[5]. TNF-α up regulates intercellular adhesion molecule-1 (ICAM-1), a transmembrane glycoprotein[6, 7] that binds to the beta-two-integrins of EPCs[8]. ICAM-1 is present on a wide variety of cells types from ECs to leukocytes[7], and its expression level depends on the concentration of pro- and anti-inflammatory mediators[7].

When EPCs are stored in the bone marrow, they are in a relatively oxygen-poor environment that ranges from 1-7%[7]. The EPCs migrate through the blood stream in response to paracrine signaling factors, and become reoxygenated until they come in contact with the wound site. Wu et al.[9] examined EPC-EC interaction in both in vitro and in vivo models through the labeling of ICAM-1 and immunofluorescent tagging of EPCs. They were able to study EPC attachment and recruitment in an ischemic myocardium murine model. They demonstrated the necessity of a hypoxic environment in the recruitment of

EPCs for proper healing. Yoon et al[10] had a similar approach and were able to show the importance of ICAM-1 in EPC recruitment in a murine model for ischemic muscle tissue. However, as EPC attachment onto the wound site occurs under blood flow in hypoxic conditions, a biomimetic model that captures the role of hypoxia and shear stress is necessary to understand the mechanisms underlying the EPC recruitment to the wound-healing site.

Microfabrication has been used to generate advanced tissue culture systems that mimic different aspects in the *in vivo* microenvironment for several years. Microfabrication has been used to generate cellular matrix's[11, 12] micropatterns of extracellular matrix protein[13, 14], and microfluidic devices [15]. In a review by Smith et al[16] the authors look specifically at how microfluidics have been used to explore vascular tissue engineering, specifically angiogenesic pathways and how the extracellular environment affect vascular tissue. The ability to control both physical and chemical cues of the environments in microfluidics make it an ideal field through which to examine the key physiological and mechanical stimuli in the circulatory system such as oxygen concentration and shear stress.

We have previously developed a microfluidic system that can adequately control oxygen tension independently from flow rate[17, 18]. This device prevents oxygen deviations along the channel, even at negligibly low shear stresses, to allow for maintaining cells in no shear conditions. Similarly, the channel height and the thickness of the upper polydimethylsiloxane (PDMS) wall were designed in a way to prevent the oxygen deviations across the channel height, allowing this system is to prevent uncontrolled oxygen gradients rather than creating them. This system also allows for long-term cell culture. In the current study, we modified the microbioreactor (MBR) system to allow for the three-

dimensional (3-D) confluent cell coverage over a uniform substrate on all sides of the MBR, while maintaining uniform oxygen concentration throughout the 3-D MBR.

Using this model we were able to study the effects of various oxygen tensions with or without TNF- α stimulation during EPC attachment to ECs. In our experiments we used endothelial colony forming cells (ECFCs) as EPCs[19, 20], and human umbilical vein endothelial cells (HUVECs) for the endothelial lining of the device. We compared the conventional two-dimensional static culture (i.e. Petri dish) and 3-D dynamic model and found that both ECFC attachment to HUVECs and ICAM-1 expression in HUVECs increase significantly when the HUVECs were cultured under 0.05 mL/H flow, 1% oxygen, and stimulated with TNF- α.

Goals

This first goal of this project was to develop a three-dimensional (3-D) microbioreactor to be used as an *in-vivo* mimicking model for vascular tissue in which oxygen tension and shear stress can be controlled independently from one another. Using this new system, we then explored EPC recruitment, attachment, and retention in a wound healing environment. We investigated the effect of hypoxia and TNF-α on ICAM-1 expression and EPC recruitment in the 3-D MBR.

Overall Approach

The newly designed 3-D MBR was created to have one substrate present on sides of the reactor. This first modification enabled the seeding of cells onto a similar substrate on all four sides of the MBR. Second, a novel three-dimensional seeding protocol was established

to efficiently and consistently allow confluent cell seeding on all sides of the micro-bioreactor. After establishing the protocol the new 3-D MBR was modeled using COMSOL to analyze the shear profile the cells experience as well as to confirm constant oxygen tension is constant throughout the MBR.

With the MBR protocol established, we explored the effects of hypoxia and TNF-α regarding EPC recruitment. ICAM-1 plays a key role in the recruitment of EPC to endothelial cells [8-10]. We performed flow cytometry, reverse transcriptase polymerase chain reaction (RT-PCR), and immunofluorescence staining and imaging to analyze ICAM-1 expression on HUVECs. HUVECs were stimulated with the following: atmospheric conditions, atmospheric conditions with TNF-α supplemented media, hypoxic conditions, and hypoxic conditions with TNF-α supplemented media. ECFCs were seeded on HUVECs in one of the four above-referenced conditions and the retention and attachment of ECFCs to HUVECs was quantified in a two-dimensional petri dish model. These experiments were then moved into the new 3-D MBR in order to properly mimic pathologically relevant conditions.

Experimental Methods

Materials

Polydimethylsiloxane

Polydimethlysiloxane (PDMS),is a silicone elastomer with many experimentally desirable properties. PDMS is chemically inert, thermally stable, and permeable to gasses[21]. One of the best benefits of utilizing PDMS is that it has the ability to fabricate microstructures. The polymer is also transparent and biocompatible.[21] In the field of

microfluidics PDMS has been developed to allow for a variety of applications: separations, patterning, and the creation of channels.[22]

Poly(methyl methacrylate)

Poly (methyl methacrylate) (PMMA) is an optically clear acrylic that has very low oxygen permeability (4.3×10^{-17} mol/s cm mmHg[23, 24]). This acrylic is used to fabricate the casing that surrounds the 3-D MBR and plays a key factor in allowing oxygen tension to be controlled independently from media flow rate.

Microfabrication of 3-D Microbioreactor (3-D MBR)

The 3-D MBR was fabricated using photolithography and soft lithography methods for the microfluidic device and machining methods for the O_2 enclosure. The channel mold was fabricated using a negative photoresist (SU8-2100, Microchem Corporation, Newton, MA, USA). The O_2 enclosure was fabricated with polydimethylmethacrylate (PMMA) sheets (9 mm thick; McMaster-Carr, Princeton, NJ, USA). A single channel fluidic device of polydimethylsiloxane (PDMS) was made to be 250 μm in width and 250 μm in height, and 2.5 cm in length. Standard photolithography techniques were used to fabricate the mold (Figure 1).

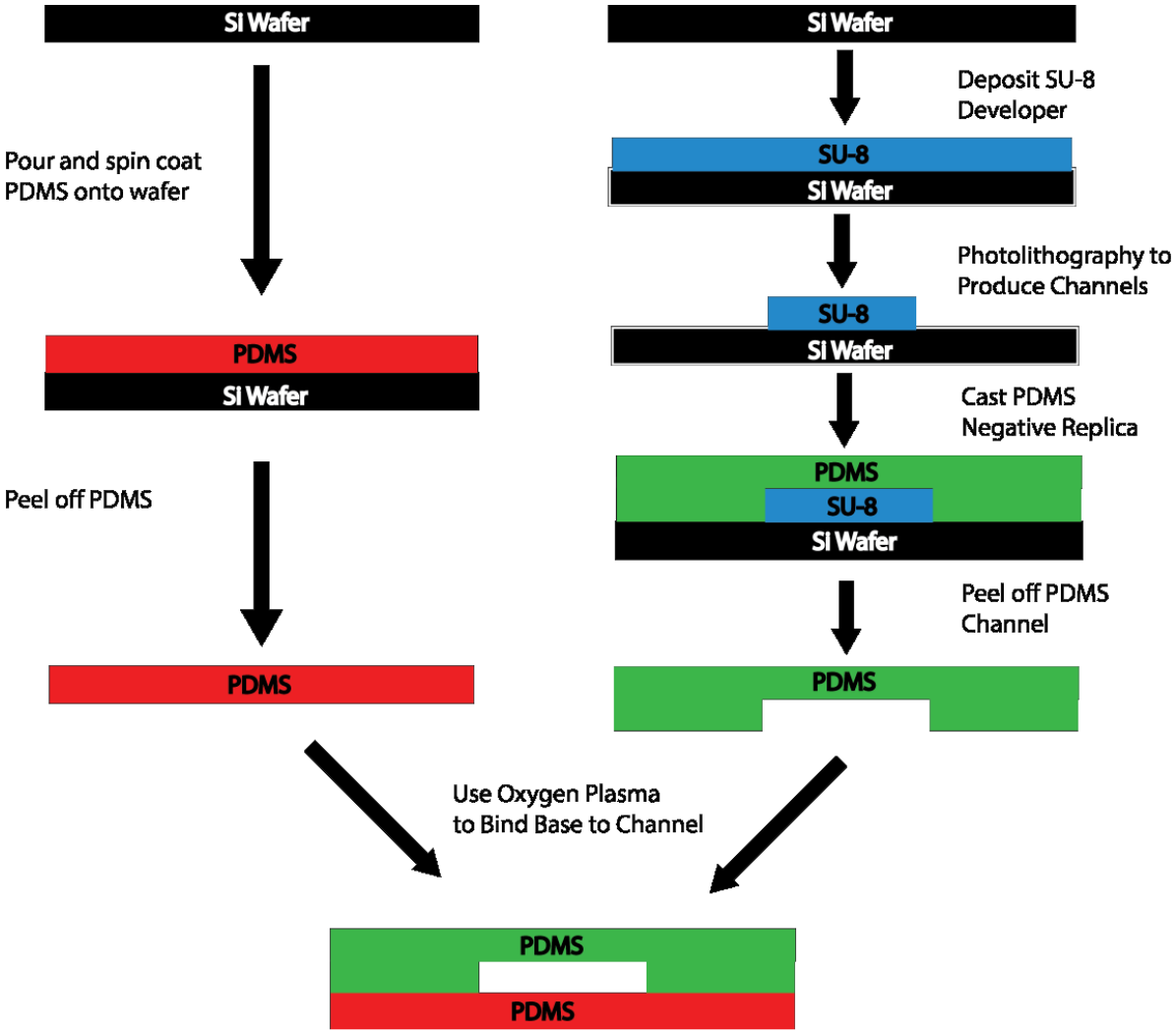

Figure 1: Microfabrication of MBR was performed using photolithography and softlithography. Photolthography was performed to create the channel mold, and softlithography was used to create PDMS replicas of the base and channel portions of the MBR. Blue is the photoresist that is used the photolithography process, green is the PDMS used to make the channel portion of the MBR, and red is used to make the PDMS used to make the base portion of the MBR.

At each end of the channel there was a 3 mm open circle port. The PDMS was spin coated (in order to control for height) and then let to cure *in situ*. An additional layer of PDMS was also spun. We peeled off the cured PDMS, punched circular holes at both ends of the channel and removed the debris through sonication with isopropyl alcohol. The punched holes were used as inlet and outlet ports for the culture channel. The base layer of PDMS was placed on the glass slide. The channel and the glass slide covered with PDMS was exposed to mild O_2 plasma and bonded together. Two additional blocks of PDMS was

partially cured using a different mixing ratio and bonded using O_2 plasma on top of the inlet and outlet holes. These PDMS blocks act as supports for the inlet and outlet tubes. The entire device was cured overnight at 65°C under weight to form a monolithic PDMS structure (Figure 2).

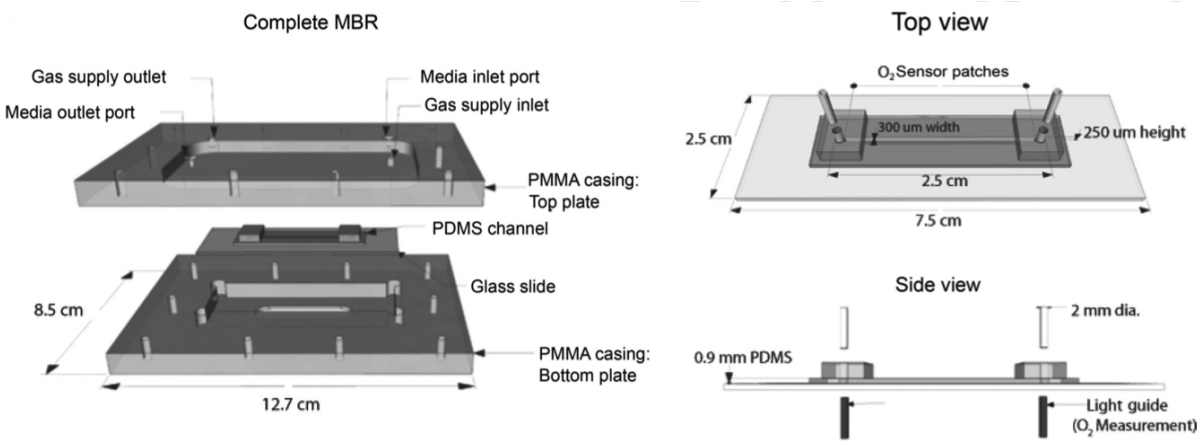

Figure 2: Microbioreactor Design: The Figure above is reproduced from Abaci et al and shows the enclosure and the microbioreactor.[17]

For the 1% oxygen experiments the microfluidic device was housed inside a PMMA enclosure. The bottom block contained a trench to hold the glass slide and has a window at the bottom so that high-magnification objectives can come in close proximity to the cells for inverted microscopy. The plates were screwed together to form an enclosure, and vacuum grease was used to make the chamber airtight.

Mathematical Model of 3-D MBR

The parameters and assumptions used in Abaci et al[17] were used in our mathematical models. The estimated wall shear stress was calculated using equation 1[17].

$$\tau_{min} = \frac{6\mu Q_{min}}{{h_{min}}^2 w} \quad (1)$$

In this equation τ_{min} is the minimum shear stress at the channel wall, μ is the dynamic viscosity, Q_{min} is the volumetric flow rate, h_{min} is the minimum height, and w is the width of the channel.

The computational fluid dynamics module in COMSOL Multiphysics® 4.4 software was used to perform the numerical calculation of the shear stress distribution and oxygen concentration in the MBR. The 3-D MBR was created in the software and was the same dimensions as the bioreactor used in the experiments. An automatic generated fine mesh divided the entire fluid body into >30,000 elements. The velocity field was calculated following the Navier-Stokes equation for incompressible fluid at steady state, neglecting gravity:

$$\rho(\boldsymbol{u} * \nabla)\boldsymbol{u} = \nabla * [-\mathrm{p}\boldsymbol{I} + \mu(\nabla\boldsymbol{u} + (\nabla\boldsymbol{u})^T)] \quad (2)$$

where ρ is the density, μ is the dynamic viscosity of the fluid, **u** is the vector of velocity in all three directions, p is the pressure, and ∇ is the gradient operator. The shear stress tensor in the X-Z direction of the plate was defined as:

$$\tau_{xz} = \mu(\frac{dv_x}{dz} + \frac{dv_z}{dx}) \quad (3)$$

For the oxygen diffusion modeling COMSOL was also used to model the oxygen concentration throughout the system. The parameters in Abaci et al[17] where used again with the addition of extra cell layers to the model. Oxygen concentration was calculated using the convection diffusion equation:

$$\frac{dc}{dt} = \nabla * (D\nabla c) - \nabla (uc) - R \quad (4)$$

where c is the concentration of oxygen, D is the diffusivity, u is the average velocity, R is your species generation term and ∇ is the gradient operator. In the mass transfer COMSOL

model a fine mesh was generated and the entire fluid body was divided into >10,000 elements. In this model we used the cellular consumption of oxygen from Abaci et al.[25]

Cell Culture

Human endothelial colony forming cells (ECFCs) (passages 6-10; Lonza, Walkersville, MD, USA) were cultured in dishes coated with type 1 collagen (Roche Diagnostics, Basel, Switzerland) in endothelial growth medium (EBM-2 Basal Medium 500 mL; Lonza, Walkersville, MD, USA) and supplemented with EGM-2 BulletKit (Lonza, Walkersville, MD, USA). ECFCs were passaged every 3 to 4 days with 0.05% trypsin (Invitrogen, Grand Island, NY, USA).

Human umbilical vein endothelial cells (HUVECs) were cultured using endothelial growth medium (EGM; PromoCell, Heidelberg, Germany). The HUVECs (passaged 3-5; PromoCell) were passaged every 3 to 4 days in 0.05% trypsin (Invitrogen).

Static ECFC Attachment Assay

HUVECs were cultured in a six well plate until they reached confluency. Each plate was then treated in one of four conditions for 24 hours. The four conditions were 21% oxygen (atmospheric), atmospheric with 10 ng/mL TNF-α[14] dissolved in the media, 1% oxygen (hypoxia), or hypoxia with 10 ng/mL TNF-α dissolved in the media. ECFCs that were cultured to confluency and treated in 1% oxygen for 24 hours were passaged and labeled using a red membrane dye (PKH26 Red Fluorescent Cell Linker Kit; Sigma-Aldrich, St. Louis, MO, USA). The ECFCs were then re-suspended in 0.1% BSA in PromoCell media and seeded on top of the HUVEC monolayer. The cells were left to statically attach for 45 minutes and then were gently washed 3 times to assess the attachment of ECFCs to the HUVECs. Some samples were further washed (10 times) to assess the retention of the

ECFCs. The plates were then imaged using light and fluorescent microscopy and merged using ImageJ. For each condition ten fields were counted, and the average number of ECFCs attached was calculated. ECFC attachment in petri dish was graphed as the number of ECFCs per well in the different conditions. A t-test was used to assess the variability in the data. The average number of cells per field was plotted for each of the given conditions.

3D Microbioreactor Preparation and Cell Culture

3-D MBR preparation was performed as previously described[17, 18]. The device was cleared with 3 mL of 70% ethanol and 6 mL of phosphate-buffered saline (PBS). 50 μg/mL of fibronectin (Fn) was flowed into the 3-D MBR manually (Sigma-Aldrich, St. Louis, MO, USA) and allowed to incubate at room temperature for 2 hours. The channel was then rinsed with PBS prior to cell seeding. HUVECs are then seeded into the 3-D MBR at a concentration of 4 million cells per milliliter by injecting through the media inlet port. The cells were then incubated for 3 hours at 37°C to promote attachment. Media perfusion was performed at a flow rate of 0.05mL/H for 24 hours. After 24 hours of media perfusion another 4 million cells per milliliter were injected through the media inlet port, and were left to attach for 3 hours at 37°C with the device placed upside-down. Media was then perfused through the device again at 0.05 mL/H for 24 hours. Finally, before proceeding with experiments the device was tested for proper cell attachment and morphology. The device also has the ability to be enclosed in an impermeable PMMA case, and the oxygen environment inside the 3-D MBR can be controlled. The PDMS acted as a highly permeable membrane and allow oxygen to diffuse through the top, bottom, and walls of the 3-D MBR independently from flow rate.

ECFC Attachment in 3-D Microbioreactor

After the 3-D MBR had been prepared it was then treated in one of two conditions for 24 hours. The first condition had complete cell media flow through the 3-D MBR at 0.05 mL/H, minimal flow rate to maintain cell culture[18], in a 21% oxygen environment (atmospheric). The second condition had complete cell media with 10 ng/mL of TNF-α flow into the device and in a 1% oxygen environment (hypoxia). ECFCs that had been pretreated in a 1% oxygen environment for 24 hours were then labeled using PKH26 red (Sigma Aldrich). The ECFC's were re-suspended in PromoCell media with 0.1% BSA and set to flow into the microfluidic device at 0.118 mL/H[26] for 1 hour. This flow rate allows for us to pathologically mimic the pressure drop of an arteriole in the MBR. Attachment was then assessed using overlapped fluorescent and light microscopy images. For each condition ten fields were counted, and the average number of cells attached on the top, bottom, and sidewalls was calculated, and is graphed as ECFC attachment to HUVECs in the microfluidic device. A t-test was used to assess the variability in the data. The average number of cells per field was plotted for each of the given conditions.

Immunofluorescence Staining and Imaging

ECFCs were labeled with PKH26 (Sigma-Aldrich) according to manufacturer's instructions. For cell fixation and staining in the 3-D MBR all occurred at a flow rate 1 mL/H. Cells were fixed by flowing 0.5 mL of 3.7% paraformaldehyde fixative and incubated for 15 minutes at room temperature. For staining cells were parabolized with a solution of 0.05% Triton-X for 10 minutes and washed with PBS. They were then incubated for 2 hours with primary antibody. The primary antibody used was for ICAM-1 (1:200; R&D Systems, Minneapolis, MN). Cells were then rinsed with PBS and incubated with anti-

mouse IgG Cy3 conjugate (1:50; Sigma-Aldrich) for 1 hour. Certain samples were rinsed with PBS and incubated with Alexa Fluor 488 Phalloidin (1:40; Invitrogen) for 30 minutes and with 4-6-diamidino-2-phenylindole, DAPI, (1:1000; Roche Diagnostics) for 10 minutes. The labeled cells were examined using fluorescence microscopy (Axiovert, Carl Zeiss, Thornwood, NY, USA).

We confirmed the uniform monolayer around the inner walls of the 3D MBR channels by confocal imaging. A z stack was taken and images were acquired every 10 μm and compiled using ImageJ to make the 3-D renderings and demonstrate uniform HUVEC coverage along the 2.5 cm length of the device.

Flow Cytometry

HUVECs were treated in one of four conditions: 21% oxygen (atmospheric), atmospheric with 10 ng/mL TNF-α dissolved in the media, 1% oxygen (hypoxia), or hypoxia with 10 ng/mL TNF-α dissolved in media. The cells were then fixed and stained for ICAM-1 with primary and secondary antibodies (as detailed above) in PBS plus 0.5% saponin (Sigma). Analysis was carried out in a FacsCalibure flow cytometer (Becton Dickinson) and using corresponding IgG controls. Data was analyzed using forward-side scatter plots to exclude dead cells. Cyflogic v1.2 was used to complete the flow cytometry analysis.

RT-PCR

A two-step reverse transcription polymerase chain reaction (RT-PCR) was performed on HUVECs treated in one of four conditions; 21% oxygen (atmospheric), atmospheric with 10 ng/mL TNF-α dissolved in the media, 1% oxygen (hypoxia), or hypoxia with 10 ng/mL TNF-α dissolved in the media. An ICAM-1 primer was analyzed

using a comparative computerized tomography method (Applied Biosystems) to calculate the amplification between the samples. B-actin was used as the reference gene. The values were then graphed and averaged with standard deviations.

Graphs and Statistics

All analyses were performed in triplicate samples for n=3 at least. Real-time RT-PCR was also performed on triplicate samples (n=3) with triplicate readings. T-Tests or One Way ANOVA with Bonferroni post-hoc test was performed where appropriate to determine significance (GraphPad Prism 4.02). Graphed data is presented as average ±SD. Significance levels were set at: $P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Results

3D Microbioreactor

The purpose of this device is to maintain the previously described characteristics of monitoring and controlling oxygen tension at the cellular level[17, 18] as well as to allow for 3-D cell coverage on a uniform substrate, permitting oxygen diffusion through all sides of the device. Figure 3 shows the modifications that were made to the MBR.

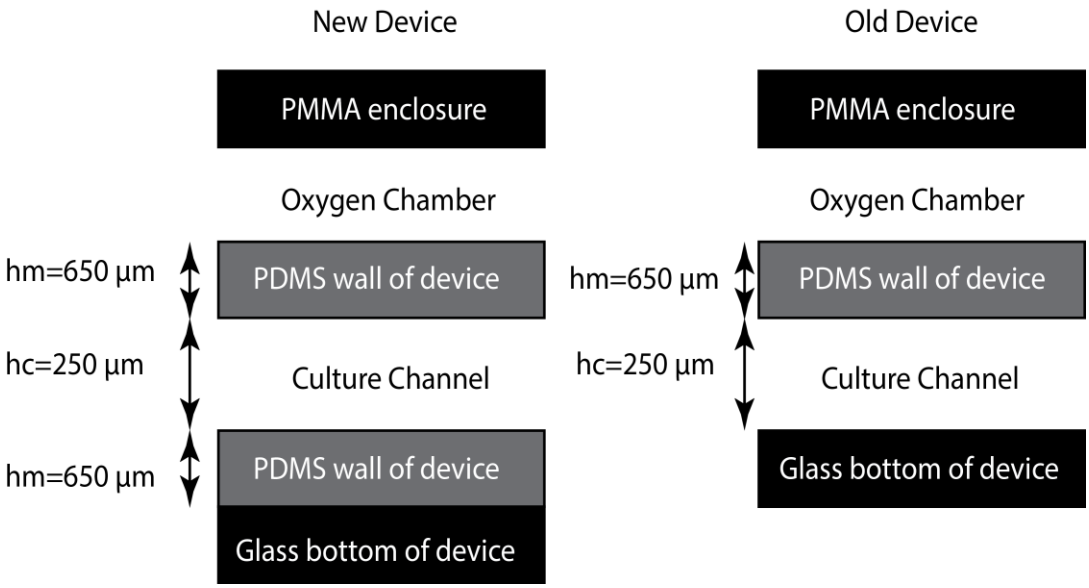

Figure 3: The modification of the extra PDMS layer to the new 3-D device: hm is the thickness of the PDMS walls (650 µm) and hc is the height of the culture channel (250 µm). The channel is also 2.5 cm long.

A rectangular channel was used for the simplicity of fabrication that is 250 µm tall (hc) and wide, as well as 2.5 cm long. The PDMS walls are 650 µm tall (hm). The use of the rectangular channel further allowed for equal coverage HUVECs with a simple seeding method, and without the use additional equipment. First, the shear stress profile was modeled using COMSOL (Figure 4).

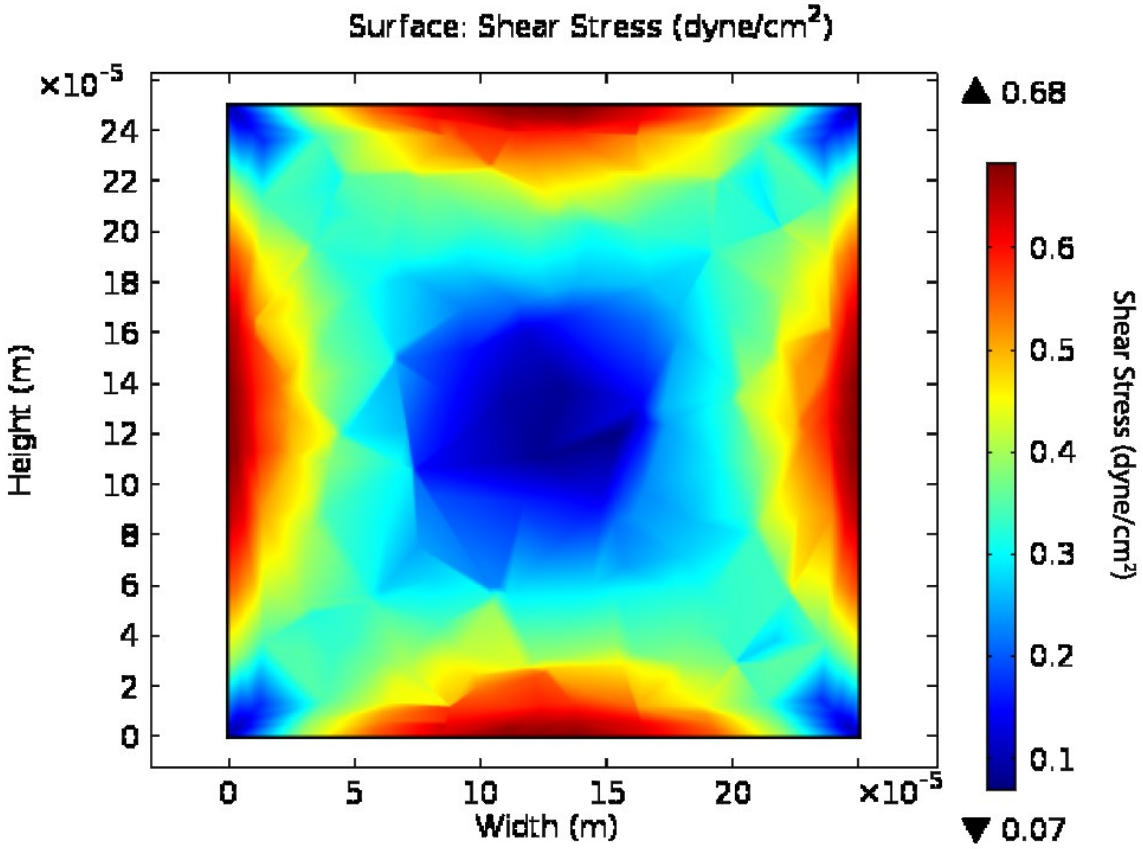

Figure 4: Shear Stress in the MBR: COMSOL was used in order to model the shear stress in the 3-D MBR at a flow rate of 0.1 mL/H.

There is a single magnitude difference between the shear at the corners and the shear felt at the center of a wall. There is a single magnitude increase in the shear stress in the middle of the channel compared to the shear stress at the walls. At the corners of the channel there is 0 dyne/cm^2 of shear. However, this only extends ~5 μm out from the corner of the MBR.

We performed computer simulations using COMSOL Multiphysics on the initial design to show that uniform oxygen distribution can be achieved throughout the channel, at various flow rates of media through the device (Figure 5A-B).

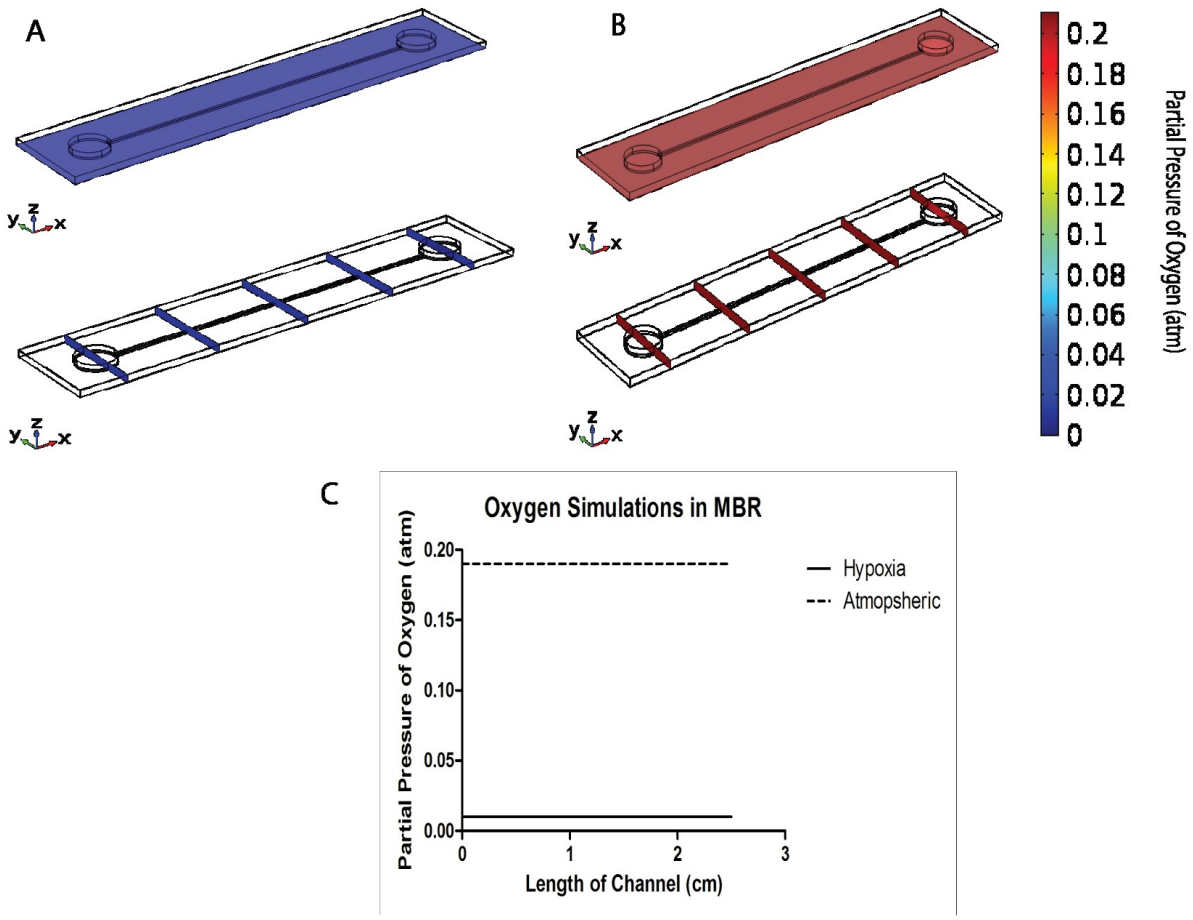

Figure 5: 3-D MBR simulations and Design. A COMSOL Multiphysics simulations showing percent the partial pressure of oxygen (atm) for the new 3-D MBR design were run with 1% oxygen (hypoxia) (A) with 21% oxygen (atmospheric) (B) around the 3-D MBR (units are in atmospheres) . (C) Graph showing the concentration of oxygen versus the different x coordinates of the channel.

This MBR allows for oxygen tension to be controlled independent of media flow rate. At both 1% O2 (Figure 5A) and 21% O2 (Figure 5B) the oxygen tension was uniform along the height and length of the channel (Figure 5C). Once the basic parameters where established we continued with cell seeding in the channel.

We further developed a novel and time-efficient seeding method to obtain a confluent monolayer of HUVECs on all sides of the micro channel (Figure 6).

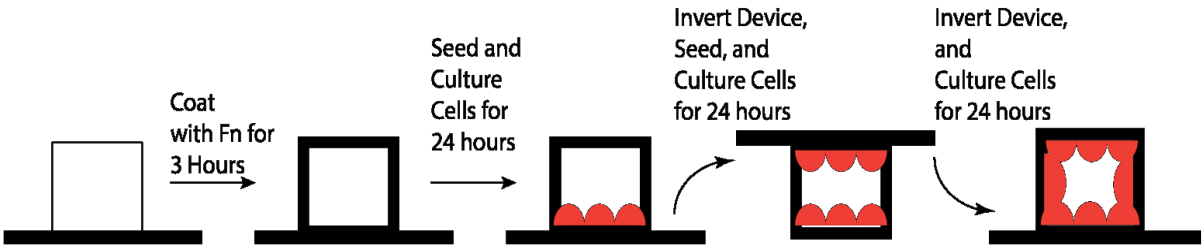

Figure 6: Seeding Protocol in MBR: Flow diagram of the seeding protocol for the 3-D MBR.

We first used an autoclaved 3-D MBR, sterilized it using ethanol, and flowed high-concentration fibronectin (Fn) into the device and let it sit statically at room temperature for 2 hours to incubate, which allowed cell attachment on all four sides of the microfluidic channel. Next we flowed in HUVECs by manual injection and let them statically attach for 3 hours to form the initial monolayer of HUVECs. The HUVECs were then cultured overnight to have the cells further anchor and spread in the device. The next day, a second seeding of HUVECs was performed in the device. The cells were flowed in at 5 mL/H using a syringe pump. The pump was used because it allowed for a quick delivery of cells without compromising the existing monolayer. We inverted the device and allowed the cells to settle and attach gravitationally. The cells were then cultured with the device inverted for 24 hours, with constant media perfusion at 0.05 mL/H. Finally the HUVECs in the 3-D MBR were cultured for a third day of constant media flow rate of 0.05 mL/H to allow cells to grow to confluency on the sides of the 3-D MBR. 0.05 mL/H is the minimal flow rate to culture HUVECs in this MBR with a height of 250 μm, as shown by Abaci et al[17]. Using this step-wise seeding approach, we achieved a quick and efficient way of having equal surface coverage of HUVECs in the 3-D MBR.

We confirmed the uniform monolayer around the inner walls of the channel by confocal imaging (Figure 7).

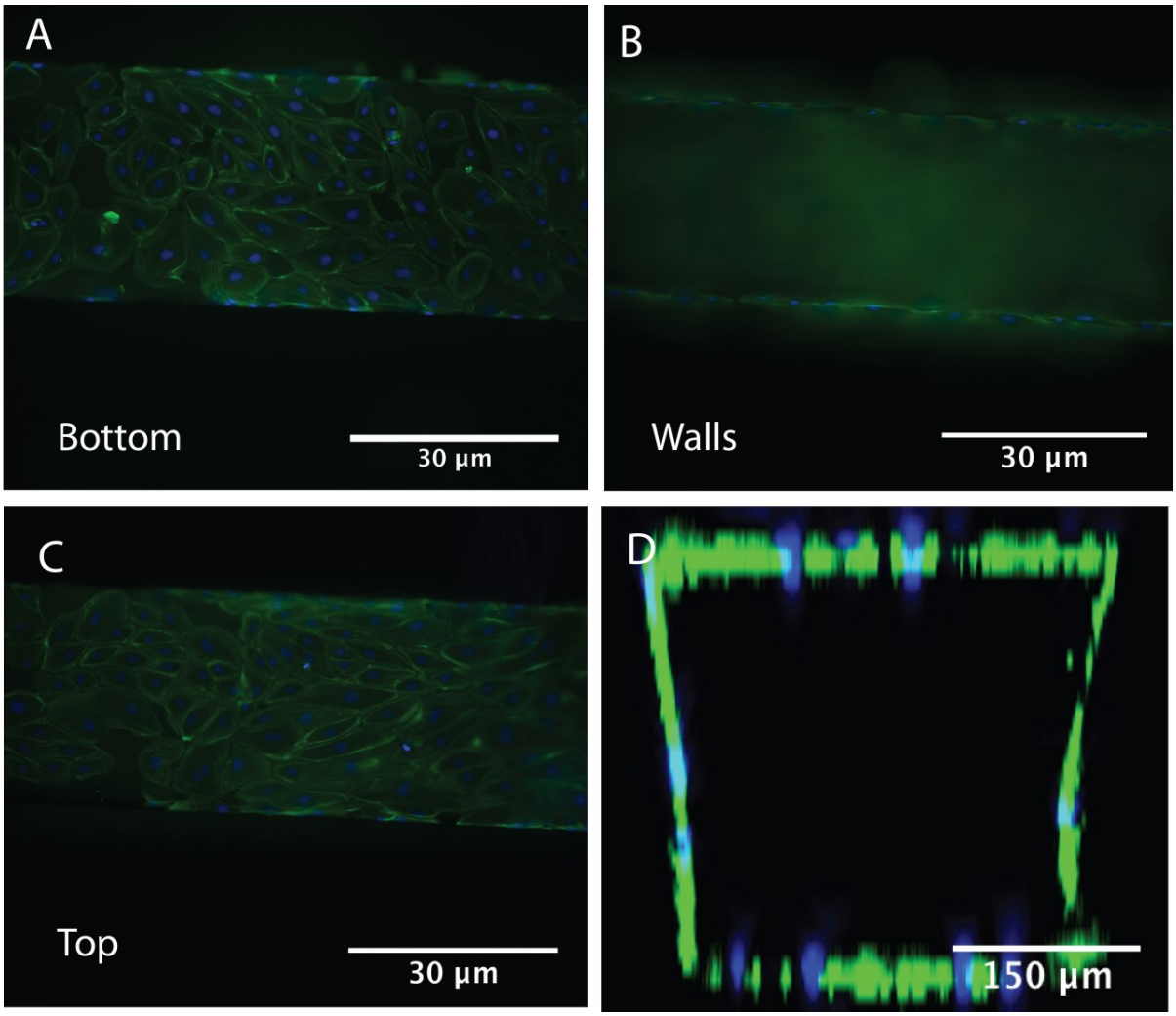

Figure 7: Immunofluorescent images: Seeding photos (phalloidin in green, nuclei in blue) show the bottom (A); side (B); top (C); and orthogonal (D).

We can see in that the bottom (Figure 7A), walls (Figure 7B) and top (Figure 7C) of the 3-D MBR were completely confluent. Orthogonal slice (Figure 7D) show the entire coverage of the device. Finally we used images that were taken every 10 µm and compiled the images using ImageJ to make the 3-D renderings (Figure 8).

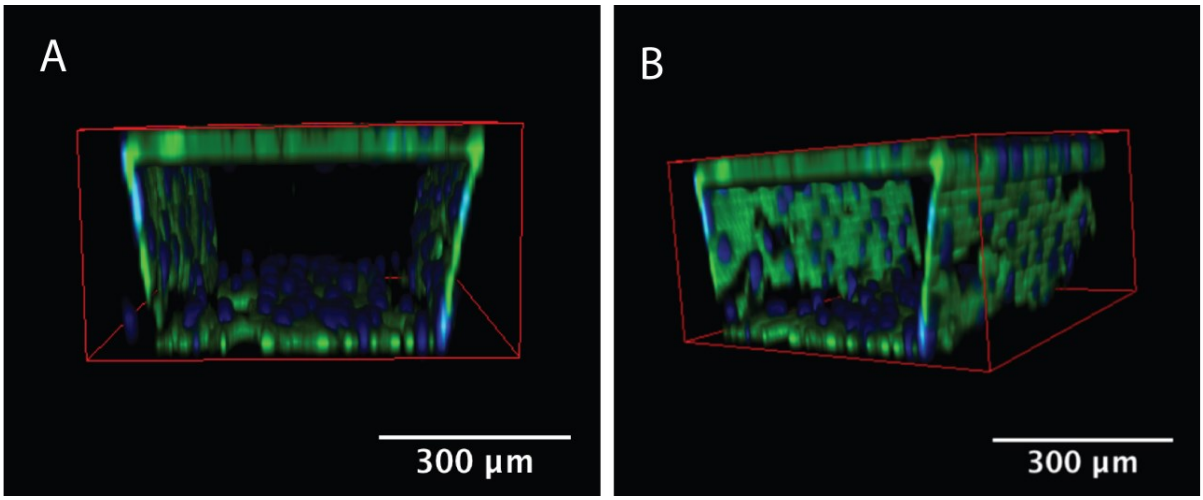

Figure 8: 3-D MBR renderings of the front (A) and cross-section (B) of the whole 3-D MBR created using ImageJ and a confocal z stack with image slices taken every 10 µm.

This demonstrates the uniform HUVEC coverage along the 2.5 cm length of the device (Figure 8A-B). Overall, the 3-D MBR device developed here allows for us to set up a pseudo-vasculature system with control over shear stress and oxygen tension.

ICAM-1 Up Regulation by Hypoxia and TNF-α Mediates ECFC Attachment to HUVECs at Static Conditions

To explore the utilization of our newly developed system to model EPC recruitment during wound healing, we first performed experiments in a static model. We cultured HUVECs on 35 mm petri dishes, coated with Fn, and allowed them to reach confluency. We hypothesized that ICAM-1 is the major adhesion molecule responsible for the attachment of ECFCs to HUVECs in wound healing model[1]. We first set out to find the optimal conditions to increase ICAM-1 levels. We cultured HUVECs in four different conditions; 21% oxygen (atmospheric), atmospheric in media supplemented with 10 ng/mL TNF-α dissolved in the media, 1% oxygen (hypoxia), or hypoxia in media supplemented with 10 ng/mL TNF-α. We found that hypoxia induces up regulation of ICAM-1 mRNA expression, as previously demonstrated [27, 28], while TNF-α supplementation profoundly increases

the expression of ICAM-1 in both atmospheric and hypoxic atmospheric conditions (Figure 9A). Specifically, HUVECs cultured under hypoxic conditions in media supplemented TNF-α showed a 1000 fold increase in mRNA expression of ICAM-1 and the protein expression increased from 27.7% ± 8.1% (atmospheric) to 92.9% ± 0.6% (hypoxic conditions in media supplemented TNF-α (Figure 9A-C).

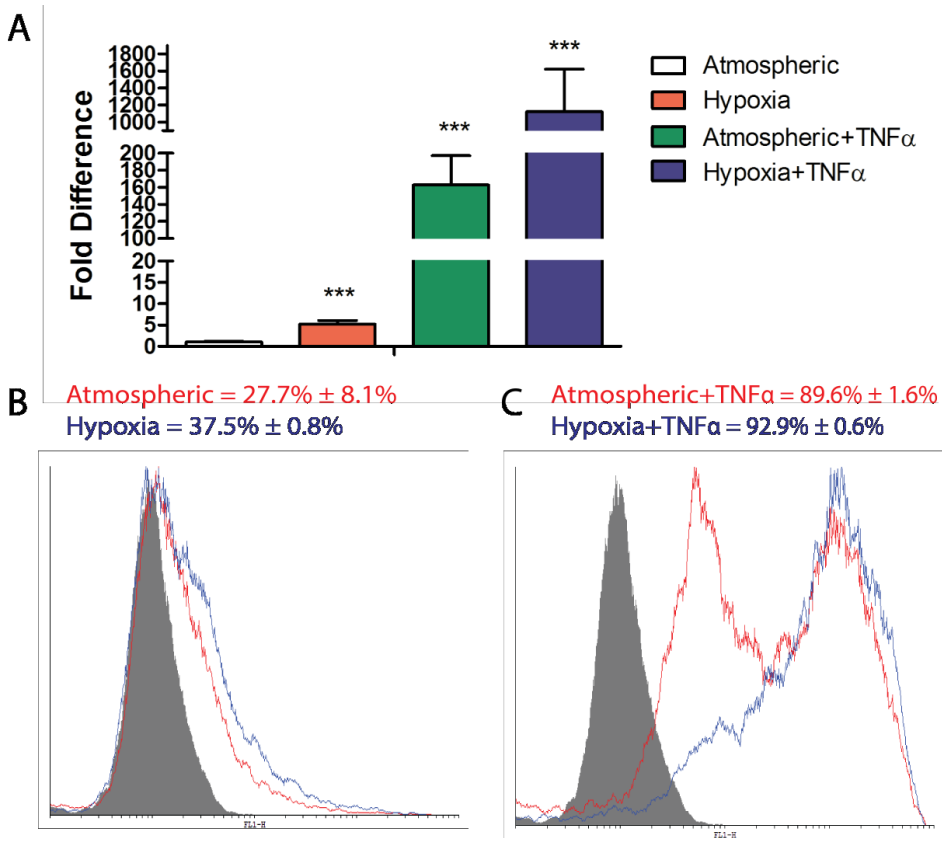

Figure 9: PCR and Flow Cytometry Data of ICAM-1 Expression in HUVECs: HUVECs were stimulated in four different conditions: 21% oxygen (atmospheric), atmospheric with TNF-α-supplemented media, 1% oxygen (hypoxia) and hypoxia with TNF-α-supplemented media and were analyzed for ICAM expression using: (A) Quantitative RT-PCR, (B-C) flow cytometry (gray histogram is IgG control). ***P<0.001.

This PCR and flow cytometry data show an up-regulation of ICAM-1 when hypoxia and TNF-α are used independently as well as synergistically. Immunofluorescent staining was performed to confirm ICAM-1 expression in the four different conditions (Figure 10A-D).

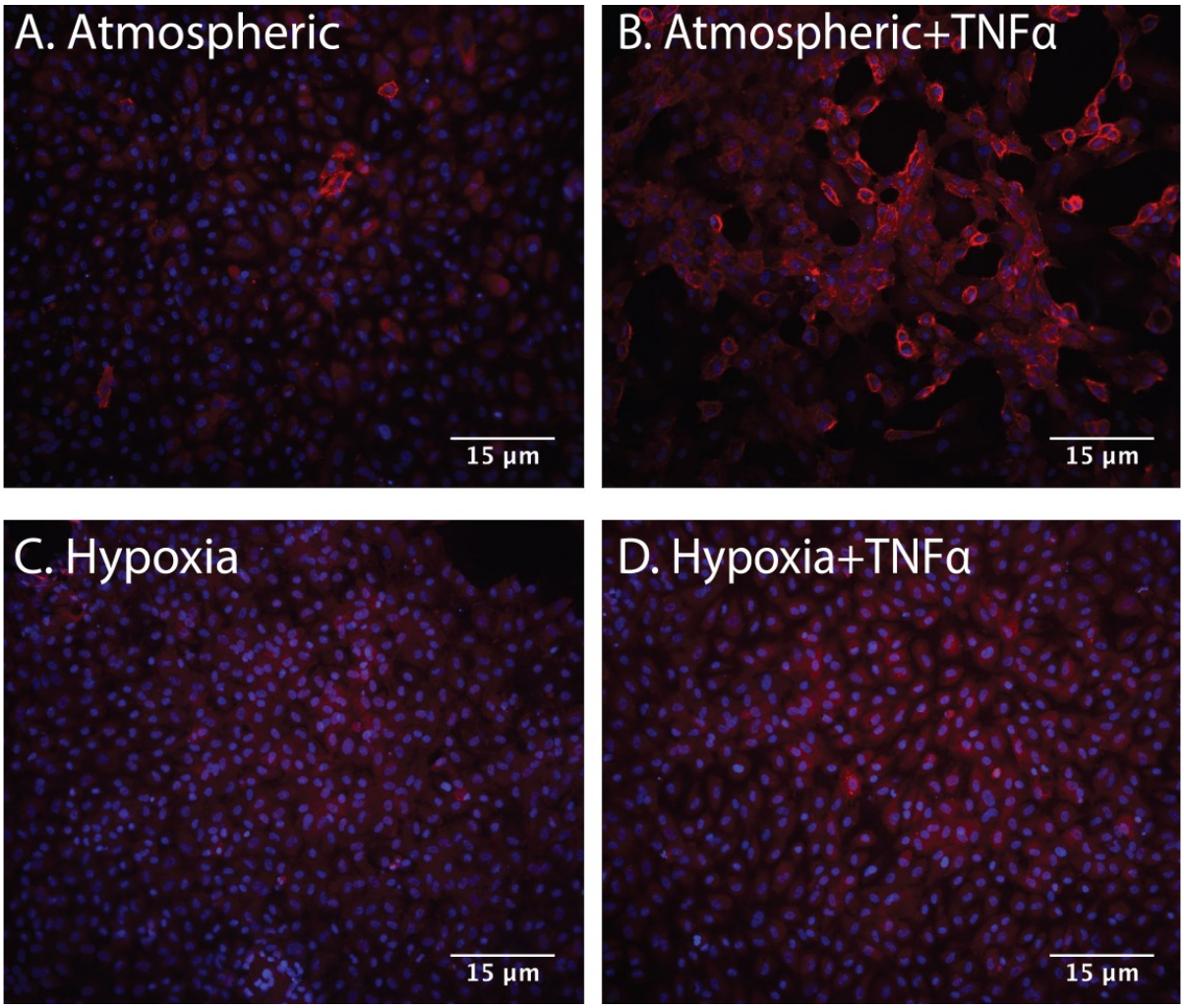

Figure 10: Immunofluorescent staining of ICAM-1 expression in HUVECs stimulated in four different conditions: 21% oxygen (atmospheric), atmospheric with TNF-α-supplemented media, 1% oxygen (hypoxia) and hypoxia with TNF-α-supplemented media (ICAM-1 in red; nuclei in blue).

From Figure 10 we can see that there is the most expression in HUVECs stimulated with both Hypoxia and TNF-α (Figure 10D), when compared to the other three conditions (Figure 10A-C). ICAM-1 is used as the intracellular binding ligand to recruit EPCs to HUVECs[9] we can hypothesize that increased levels of TNF-α expression and lowered levels of oxygen at the site of wound healing may increase EPC attachment through the stimulation of ICAM-1.

ECFCs attachment in static conditions

To test this hypothesis, we examined ECFCs attachment onto HUVECs that were treated in one of the four conditions listed above. Figure 11 shows the flow diagram of the experimental set up.

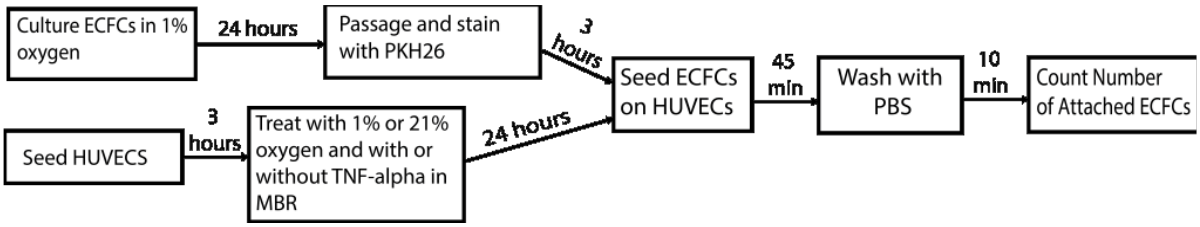

Figure 11: ECFC attachment protocol: Schematic of the experimental procedure and timeline of experiments performed in 2-D static conditions.

ECFCs were pretreated in 1% oxygen (hypoxia) for 24 hours to mimic the physiological environment in the bone marrow, where these cells reside before they enter the blood stream[29]; ECFCs were then labeled (using red PKH26 dye), seeded and allowed to attach for 45 minutes, washed and then evaluated for adhesion to the HUVECs. Using red PKH26 dye we can identify and count the number of ECFCs attached to the HUVECs. We first found that there is a clear increase in ECFC attachment to HUVECs, when HUVECs were stimulated with TNF-α, hypoxia, or both. In fact, there are double the amount of ECFCs attached when HUVECs were stimulated with TNF-α and hypoxia in comparison to the atmospheric HUVECs (Figure 12A-E).

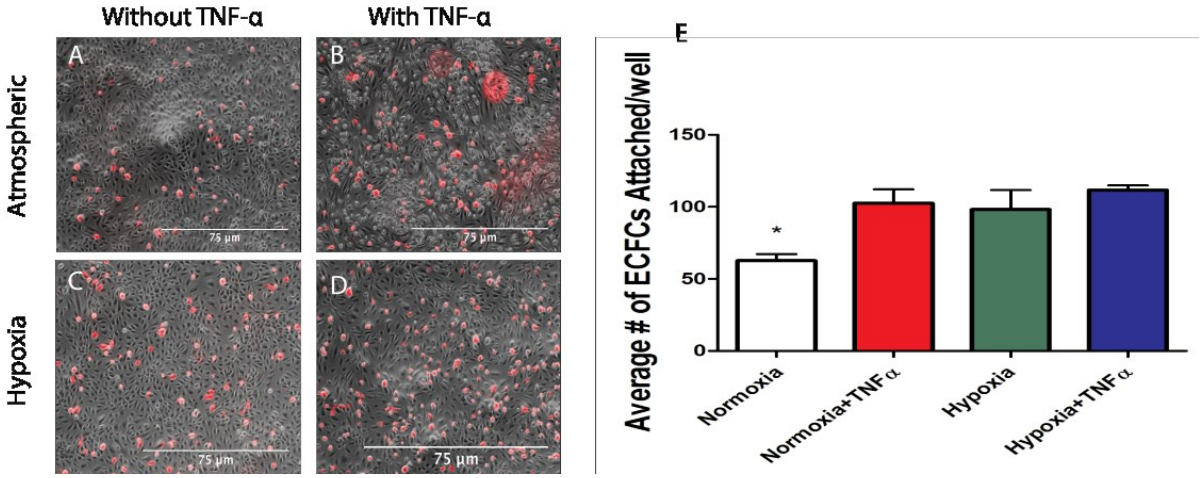

Figure 12: ECFC attachment to HUVECs: (A-D) Fluorescence images overlapping light microscopy images and (F) quantification of ECFCs (red) attached onto HUVECs stimulated in one of the four different conditions. This was done with 3 washes to look at the attachment of ECFCs to HUVECs. P<0.05.

We also found an 80% ± 9% increase in attachment of ECFCs when they are seeded on HUVECs stimulated with hypoxia and TNF-α, compared to untreated (p value<0.01) HUVECs.

For the EPCs to contribute to the vessel formation, they need to attach to the EC lining but then also need to remain attached and resilient to the shear forces applied by blood flow. Therefore, we also evaluated the number of ECFCs that were more strongly attached on HUVECs in all conditions when washed. We found that HUVECs stimulated with both TNF-α and hypoxia had the best retention of ECFCs (Figures 13A-E).

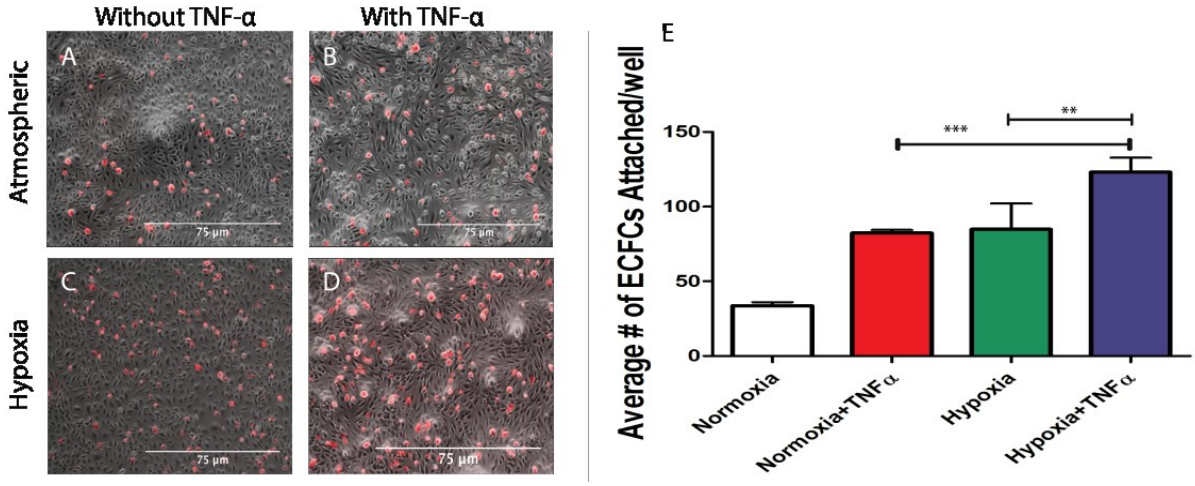

Figure 13: ECFC retention to HUVECs: (A-D) Fluorescence images overlapping light microscopy images and (E) quantification of ECFCs (red) attached onto HUVECs stimulated in one of four different conditions after extended washing period. P<0.05; **P<0.01; ***P<0.001.

There was a three-fold increase in attachment of ECFCs on HUVECs stimulated with hypoxia and TNF-α compared to atmospheric conditions. There was also a 48% ± 4.8% increase in ECFC attachment on HUVECs stimulated with only TNF-α and there was a 48% ±20% increase in ECFC attachment on HUVECs stimulated with only hypoxia (Figure 13E). From this data, we observed a strong correlation between the increase in ICAM-1 levels and the attachment and retention of ECFC in response to varying conditions. From these two-dimensional analogues we moved to the 3-D MBR. The 3-D MBR system is a useful tool to study the attachment and retention of ECFCs simultaneously at precisely controlled oxygen tension and shear forces.

ECFC attachment on HUVECs in 3-D MBR

Finally, we examined whether our newly developed 3-D MBR better mimics the wound healing microenvironment. Following the protocol described above (Figure 6), HUVECs were seeded in the 3-D MBR (as detailed in Figure 7 and 8) and grown in atmospheric conditions or stimulated with hypoxia and TNF-α supplemented media for 24 hours at 0.05 ml/H. 0.05 mL/H is what was shown by Abaci et al, as the minimum flow rate

to sustain HUVEC survival in a MBR with this height[17]. ECFCs were pre-treated in 1% oxygen for 24 hours and then labeled with PKH26 red prior to being flowed through the 3-D MBR for 45 minutes at 0.118 mL/H [26] and in 21% oxygen. That flow rate was chosen to mimic the normal pressure drop that is seen in an arteriole of this size [26]. 0.118 mL/H is used to mimic the impaired blood supply normally observed at the wound site. Using the equations in Abaci et al, we have an average shear stress 0.1 dyne/cm^2. [17]After the 45 minutes the cells were imaged and counted as above. Having a constant flow rate of 1% oxygen into the PMMA chamber that is housing the MBR oxygen tension can be controlled. This constant flow of 1% oxygen has previously been used by Abaci et al to maintain oxygen tension in a similar device[17]. Using this bombardment method we can prevent oxygen gradients from forming in the MBR, and insure that there is uniform oxygen tension.

We found that ECFC attachment to HUVECs in the 3-D MBR in atmospheric conditions was minimal (Figure 14A-C), with approximately the same amount of ECFCs attached to the top and bottom of the 3-D MBR (Figure 14C).

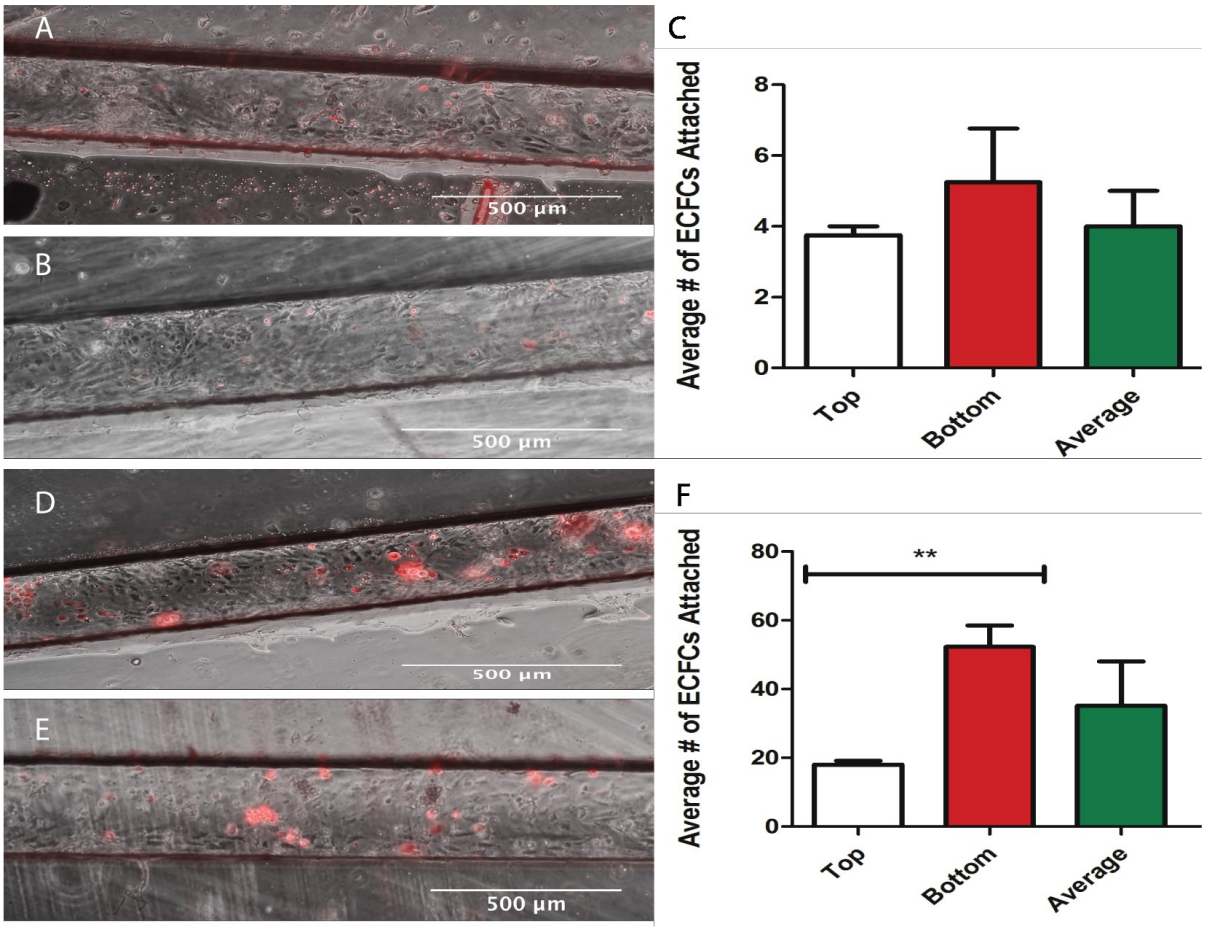

Figure 14: ECFC Attachment and retention on stimulated HUVECs in the 3-D MBR. ECFCs (red) were seeded on HUVECs stimulated in 21% oxygen (atmospheric) and imaged at the bottom (A) and top (B) of the device and quantified (C). ECFCS were also seeded on HUVECs stimulated in hypoxia with TNF-α-supplemented media and imaged at the bottom (D) and top (E) of the device and quantified (F). P<0.05; **P<0.01; ***P<0.001.

There was an increase in the average amount of ECFCs that were able to attach to HUVECs once they were stimulated with hypoxia and TNF-α at the top of the device (Figure 14D-E). Quantitative analysis revealed a ten-fold increase in the amount of ECFCs attached to the bottom of the device, and a ten-fold increase in the average number of ECFCs attached to the HUVECs stimulated with hypoxia and TNF-α when compared to atmospheric HUVECs (Figure 14C, F). Moreover, in the hypoxic conditions with TNF-α, we observed preferential attachment of the ECFCs to the bottom layer of the microfluidic device compared to the top (Figure 14F). Due to the shear force acting on ECFC during the flow experiments, the 3-D MBR model takes into account both the attachment and retention of cells simultaneously,

and as such is more accurate than a static model. Thus, the 3-D MBR allows for the assessment of ECFC recruitment in a more pathologically relevant microenvironment.

Discussion

The newly developed 3-D MBR allows for an *in-vivo* mimicking system. We first augmented the existing MBR reactor design by adding a base layer of PDMS (Figure 3). This base layer allows for the seeded ECs to be exposed to the same substrate on all sides of the system. This approach generated a rectangular channel, which simplified the fabrication. With this change we modeled the shear stress profile within the channel (Figure 4). We found that due to the rectangular design of the MBR there is a non-uniform shear stress at the corners. The shear stress at the walls of the system with a volumetric flow rate of 0.1 mL/H is at 0.63 dynes/cm^2, which is at pathological relevance for an ischemic wound. This allows us to properly mimic the flow rate and shear conditions of an ischemic wound. In a circular channel we also see a ten fold increase in the shear stress between the wall and the center of the channel (data not shown). However, at 0.1 mL/H a lower shear stress was achieved along the walls of the channel (0.1 dynes/cm^2), which is not pathologically relevant when compared to a square channel.

In most conventional *in-vitro* culture systems, oxygen concentration is linked directly to the volumetric flow rate of the liquid flown through the 3-D MBR[30]. With the addition of a PDMS base layer to the rectangular 3-D MBR and a 3-D seeding of ECs, a COMSOL model was developed to study system-wide oxygen tension. As shown in Figure 5, we found that the 3-D MBR allows for the precise control of oxygen tension and maintenance of uniform oxygen concentration along the length of the channel.

A new 3-D cell seeding protocol was also developed, which allowed for the complete coverage of the 3D-MBR. In our new approach we were able to efficiently seed HUVECs using a multistep seeding processes (Figure 6), which produces a fully confluent 3-D MBR in 3 days. This new system can be used to model different disease and injury states in order to study mechanisms and test the effectiveness of various drugs.

Using this newly developed 3-D MBR we studied what factors contribute to the recruitment of circulating EPCs to ECs. ICAM-1 is the intracellular binding ligand that recruit EPCs to ECs[9]. Thus, we first analyzed ICAM-1 expression in HUVECs cultured in Petri dishes through RT-PCR, flow cytometry, and immunofluorescent staining. We cultured HUVECs in four different conditions; 21% oxygen (atmospheric), atmospheric in media supplemented with TNF-α dissolved in the media, 1% oxygen (hypoxia), or hypoxia in media supplemented with TNF-α. We found that hypoxia induces up regulation of ICAM-1 mRNA expression, as previously demonstrated [27, 28], while TNF-α supplementation profoundly increases the expression of ICAM-1 in both atmospheric and hypoxic atmospheric conditions. These trends hold true for both the RT-PCR and flow cytometry data, and the immunofluorescent images confirm these findings.

We next hypothesized that increased levels of TNF-α expression and lowered levels of oxygen at the site of wound healing would increase ECFCs attachment through the stimulation of ICAM-1. We initially tested this hypothesis in petri dishes to assess the role of ICAM-1 in the adhesion of ECFCs to HUVECs. In this model we pre-treated the ECFCs in 1% oxygen to better mimic physiological conditions, while the HUVECs were treated in one of the four conditions mentioned above. The ECFCs were left to attach for 45 minutes and then washed with PBS. From this data we can see that TNF-α and hypoxia equally

increase the average number of ECFCs attached onto HUVECs. We also noted a slightly greater number of ECFCs attached when hypoxia and TNF-α were used together.

However, when EPCs are released into the blood they need to attach to the site and then, furthermore, need to remain attached. From the experiments above it is clear that hypoxia and TNF-α used together increase attachment. However, we wanted to explore the strength of the attachment. We performed the same experiment above, except we increased the number of times the ECFCs were washed with PBS. From this retention trial we saw a greater number of ECFCs attached when HUVECs were stimulated with hypoxia and TNF-α when compared to other conditions, suggesting a stronger attachment under this condition.

Our results are consistent with the previously shown role of ICAM-1 in EPC attachment[9]. In Wu et al., the authors showed that ICAM-1 is essential in EPC attachment through CD18 knockdown. They also showed that when CD18 is knockdown in vivo there is a 4-fold decrease in the amount of EPCs present. Carmona et al used an activated cAMP as a stimulator of ICAM-1[31] in order to increase EPC attachment and found a 50% ± 10% increase in baseline from their non-treated control[32]. In our study, when the HUVECs were stimulated with hypoxia, instead of cAMP, there was 154%± 34% increase of ECFC attachment compared to atmospheric HUVECs; and when HUVECs where stimulated with hypoxia and TNF-α there was a 272% ± 13% increase when compared to HUVECs in atmospheric conditions. In Carmona et al, also found that HUVECs treated with cAMP and TNF-α had a 50% ± 12% increase in the number of EPCs attached compared to pretreated with TNF-α only [32]. When this is compared to our results, we see stimulating HUVECs with TNF-α and hypoxia, there is a 50%±13% increase in ECFC attachment when compared to HUVECs just stimulated with TNF-α (Figure 13E). Although we were able to evaluate

the retention of ECFCs by mimicking shear with extended PBS washing, it should be noted that this method does not allow for accurately control of the shear forces applied on the ECFCs. This lead us to hypothesize that shear stress could play a role in assessing the attachment strength of ECFCs. The 3-D MBR system is a useful tool to study the attachment and retention of ECFCs simultaneously at precisely controlled oxygen tension and shear forces.

In the 3-D MBR, HUVECs were stimulated with atmospheric conditions or with hypoxia and TNF-α supplemented media. In this model we found a significant increase in the number of ECFCs attached when the HUVECs were stimulated with hypoxia and TNF-α. HUVECs stimulated in atmospheric conditions had an average of 4 ± 2 ECFCs attached, while HUVECs stimulated with hypoxia and TNF-α had an average of 35 ± 22 ECFCs attached. We also observed a preferential attachment of ECFCs to the bottom of the device, when compared to the top. In the atmospheric conditions there was a slight increase in the number of ECFCs attached to the bottom when compared to the top (Figure 14). However, HUVECs treated with hypoxia and TNF-α had two and a half times the number of ECFCs attached (Figure 14). The preferential attachment to the bottom of the channel is most likely due to gravity and the height of the channel. Due to the shear force acting on ECFCs during the flow experiments, the 3-D MBR model takes into account both the attachment and retention of cells simultaneously, and as such is more accurate than a static model. Comparing the 3-D MBR model (Figure 14) to the static 2-D, petri-dish model (Figure 13), we note that there were fewer ECFCs attached in the dynamic flow system. This phenomenon suggests fluid flow may play a role in the attachment of ECFCs during

recruitment to the wound site. Using the 3-D MBR we were able to better study the pathological environment of EPC attachment to ECs.

Conclusion

We were able to design and fabricate an improved MBR for the purpose of 3-D vascular modeling. The novel seeding method developed here allows for a quick and effective approach to generate a HUVEC monolayer on the top, bottom, and sidewalls of the device. In our model we control oxygen tension independently from flow rate. In our 3-D MBR system we were able to create an environment in which the ECs are exposed to the same substrate at all surfaces, and have sufficient oxygen diffusion on all sides of the device. These changes to the previous MBR design[17, 18] make the new 3-D MBR more pathologically relevant. We utilized the capabilities of the 3-D MBR system to study the recruitment of ECFCs in a wound-healing microenvironment. We first determined the optimal condition in which ICAM-1 is up regulated and then applied those conditions to the 3-D MBR. When HUVECs were stimulated with hypoxia and TNF-α we found a significant increase in the number and retention rate of ECFCs. Importantly, ICAM-1 expression and ECFC attachment are further up regulated when TNF-α and a hypoxic environment. The 3-D MBR allows for flow to be applied in the wound healing model and the ability to analyze the distribution of ECFC attachment when placed under shear stress. All dimensions of the system allow uniform oxygen tension at cellular level. Standard PDMS thicknesses and channel heights used in typical microfluidic systems would not allow this level of oxygen control. A limitation of the system is that the HUVECs aren't pretreated with shear stress, which would more closely mimic the endothelial lining of the blood vessels. However, using

the same substrate on all sides of the 3-D MBR and the novel 3-D seeding method allow for a more pathologically relevant system, which can be used for *in-vivo* mimicking. This system can be used as a step between traditional *in-vitro* and *in-vivo* experimentation to model a variety of vascular-related disorders, especially wound healing.

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Curriculum Vitae

Daniel Lewis

School Address
3100 Saint Paul Street, Apt 206
Baltimore, MD 21218
203-940-3237
dmlewis1234@gmail.com

Permanent Address
95 Winthrop Drive
Riverside, CT 06878

Education

Johns Hopkins University — Baltimore, MD
- Masters of Science in Chemical and Biomolecular Engineering — May 2015
- Bachelors of Science in Chemical and Biomolecular Engineering — May 2014
  - Minor in Entrepreneurship and Management

Awards

Honored for Excellence in Research by the Chemical and Biomolecular Engineering Department — 2012- 2014

Chemical and Biomolecular Engineering Award for Excellence in Research — May 2013

Provost Undergraduate Research Award — May 2012 - April 2013

Professional Experience

Lab Assistant — Baltimore, Maryland
Gerecht Lab, Johns Hopkins University — January 2011- Present
- Developed novel co-culture system to analyze interactions between endothelial and breast cancer cells
- Created a microfluidic model to explore wound healing and to enhance endothelial cell repair and recruitment
- Integrated the ability into measure impedance into existing microfluidic device in order to quantitatively and qualitatively assess the side effects of different therapies on endothelial cells

Senior Associate of Therapeutic Research — New York, New York
Leading BioSciences Inc — May 2014 – August 2014
- Established project management protocol for running a phase 2 clinical trial
- Identified the top critical care physicians to establish a phase 2 clinical trial protocol
- Wrote and helped complete sections for an Investigation New Drug Filing

Associate of Therapeutic Research — New York, New York
Leading BioSciences Inc — December 2013 – February 2014
- Generated market strategy and analysis of critical care standard of care
- Restructured clinical trial strategy off of market analysis
- Leveraged other aspects of the therapy to explore new therapeutic areas using the company's drug development pipeline

Rotational Program Intern New York, New York
Mesoblast, Ltd June 2013 – August 2013

- Rotated through Mesoblast's clinical, non-clinical, project management, quality, manufacturing, and regulatory departments to assist in the development of new stem cell therapies
- Edited and reviewed a European Medical Agency submission for a phase 3 clinical trial
- Created project management standard operating procedures for the data management division of Mesoblast
- Leveraged other aspects of the therapy to explore new therapeutic areas using the company's drug development pipeline
- Collaborated and coordinated evaluation of possible contract research organizations for new clinical programs

Clinical Practice Manager Greenwich, Connecticut
Diagnostic and Medical Specialist of Greenwich May 2011 – August 2011

- Optimized patient inflow and outflow through the development of standard operating procedures to maximize the number of patients seen by general practitioners

Publications

1. **Lewis DM**, Abaci HE, Gerecht S. Endothelial Progenitor Cell Recruitment in a Wound Healing Microfluidic Vascular Model. (Submitted 2014)
2. Dickinson LE, Lütgebaucks C, **Lewis DM**, Gerecht S. Patterning microscale extracellular matrices to study endothelial and cancer cell interactions in vitro. Lab Chip, 2012. 12(21): p. 4244-4248.

Presentations

Provost Undergraduate Research Award Ceremony Baltimore, MD
Johns Hopkins University April 2013

- Lewis DM, Abaci HE, Gerecht S. "Endothelial Progenitor Cell Recruitment in a Wound Healing Microfluidic Vascular Model."

Synergistic Activities

Institute for NanoBioTechnology Undergraduate Research Leader Baltimore, Maryland
Johns Hopkins University March 2013- May 2014

- Chosen representative to lead events to better the undergraduate research experience
- Communicated ideas from meetings to other undergraduates in the Gerecht Lab, and to Dr. Gerecht

AICHE/SBE Member Baltimore, Maryland
Johns Hopkins University September 2010 – May 2014

- Participates in biweekly in chemical and bioengineering events

Scholarships
Joseph F McElvery Memorial Award
American Red Cross Scholarship in Memory of Dwight H. Renfrew III and John E. Schmeltzer IV Award
Friends of Greenwich Point Award
Greenwich High School PTA Council Award
Charlton Fund
Countess Frances Thorley Palen-Klar Scholarship Fund