Design and use of an environmental control platform for studying vascular cell function in three-dimensional scaffolds

by

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Abstract:

Endothelial and smooth muscle cells are the core of the vascular system. Vessels are created and repaired through the processes of angiogenesis and arteriogenesis. Endothelial cells are the initial cells that migrate and proliferate during the process, followed by smooth muscle cell movement and differentiation. The recruitment of smooth muscle cells is not fully understood, and if understood would unlock a crucial step in the growth and remodeling of vessels.

Endothelial and smooth muscle cells have been extensively studied in two dimensional models and the physiologic factors that affect their function and survival have been well documented. In living organisms, vascular tissue, consisting primarily of endothelial and smooth muscle cells, grows in three dimensions where it is constantly exposed to biochemical and biomechanical stimuli. Thus, a controllable three dimensional environment is desired to study these interactions. The cells do not move instantaneously or in direct paths, hence, it is beneficial to be able to study the cells at multiple time points and over long periods of time. Most *in vitro* studies have not been conducted for more than 100 hours, while *in vivo* experiments have been continued for months. To fully understand *in vitro* growth of smooth muscle cells, the growth should be monitored continually and the observation technique must be able to support to this.

Accordingly, we have developed a microscope stage environmental chamber that houses a three dimensional bioreactor for vascular tissue engineering in order to monitor tissue function in real-time. We have demonstrated the capabilities of the environmental control chamber by growing smooth muscle precursor cells (10T1/2 cells) in the three dimensional bioreactor and monitored the cells for growth, migration and proliferation. Critical to the chamber design was the control of temperature, carbon dioxide concentration, and humidity.

Furthermore, smooth muscle precursor cell (10T1/2 cell) migration and morphology was observed in response to varying concentrations of Platelet Derived Growth Factor-BB (PDGF-BB), an endothelial cell-derived growth factor that is important for smooth muscle recruitment to remodeling blood vessels. Using a migration assay technique that observes the general trends of cell movement through the device, the cells exposed to PDGF-BB have been noted to move more than the cells grown in media without a growth factor. The cells tend to migrate towards the PDGF-BB with great variability.

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Chapter 1: Introduction

1.1 Vascular formation and remodeling

Endothelial and smooth muscle cells are the core of the vascular system. Vessels are created and repaired with these two cell types through normal and pathological processes of angiogenesis and arteriogenesis. These two processes are crucial to the growth and sustainability of the body. The cells and mechanisms involved have been studied in depth, but are not fully understood. Mural cells, the precursors to smooth muscle cells, are recruited to cover a preexisting network of endothelial cells created during angiogenesis. The differentiated mural cells, or smooth muscle cells, cover the structure with a muscular coat, giving the vessel its viscoelastic properties, structural integrity, and ability to constrict or dilate. A depiction of the layers of a vessel can be found in Figure 1. The environment in which these cells thrive and function continues to be studied in the context of vascular tissue engineering. Signaling molecules, such as growth factors, and mechanical forces are critical for determining these cellular functions. [1]

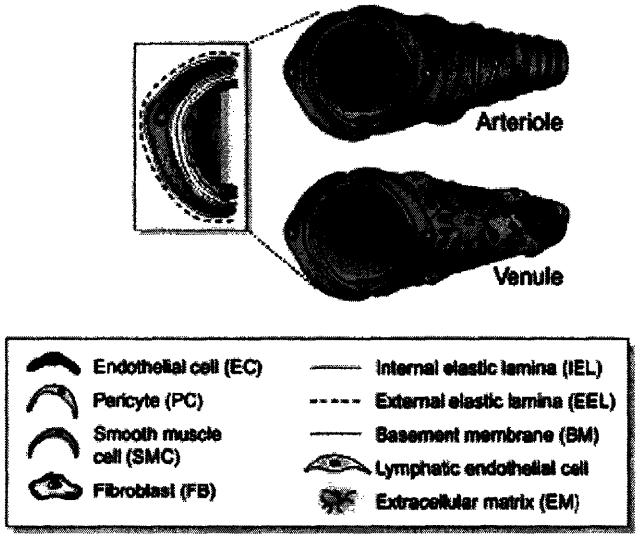


Figure 1: Depiction of the cellular structure of the vein and artery. [1]

Endothelial cells recruit and differentiate smooth muscle precursor cells during vessel maturation and remodeling through signaling pathways involving growth factors such

as Platelet Derived Growth Factor β (PDGF-β) and Transforming Growth Factor β (TGF-β).[2] Different biochemical and biomechanical cues control different aspects of vessel development and both endothelial and smooth muscle cells are involved in the process. In the specific case of arteriogenesis, arterial obstruction caused by a stenosis leads to an increase in the pressure gradient across the existing collateral vessels both because of the increased local resistance and peripheral vasodilation. This increase in pressure drives an increase in flow and consequently the wall shear stress which activates endothelial cells and recruits smooth muscle cells. The vessel enlarges, initially due to smooth muscle relaxation, but later due to active remodeling of the extracellular matrix and smooth muscle cell recruitment. If the fluid shear stress is reduced, the reverse process takes place and vessels narrow. Several endothelial and inflammatory cell factors have been associated with the optimization of this process,[3] but the process of collateral vessel development remains poorly understood.

Previous research that combines smooth muscle and endothelial cells has primarily been done in two dimensions. Smooth muscle cells and their precursor cells, 10T1/2 cells, have been grown on multiple 2D sandwich structures of collagen combined with endothelial cells. In the presence of endothelial cells, 10T1/2 cells migrate towards the endothelial cells, and when there is physical contact between the cells, the 10T1/2 cells differentiate into smooth muscle cells.[4] Smooth muscle cells and endothelial cells have also been studied under shear stress in a two-dimensional environment. The migration of the smooth muscle cells increases until the shear stress reaches 1.5 Pa. After this point, the cell migration is halted and is less than the control rate of migration. This suggests a direct relationship between stress and the remodeling of vessels.[5] These 2D experiments, however, are a poor representation of the true 3D environment in which these processes occur *in vivo*. For this reason, we have developed a 3D system that more realistically portrays *in vivo* conditions.

1.1.1 Three dimensional platform for studying vascular formation

It is now widely recognized that most cell types function differently in three dimensional environments compared to two dimensional environments. Accordingly, the vascular research conducted in the Center for Biomedical Engineering is primarily done

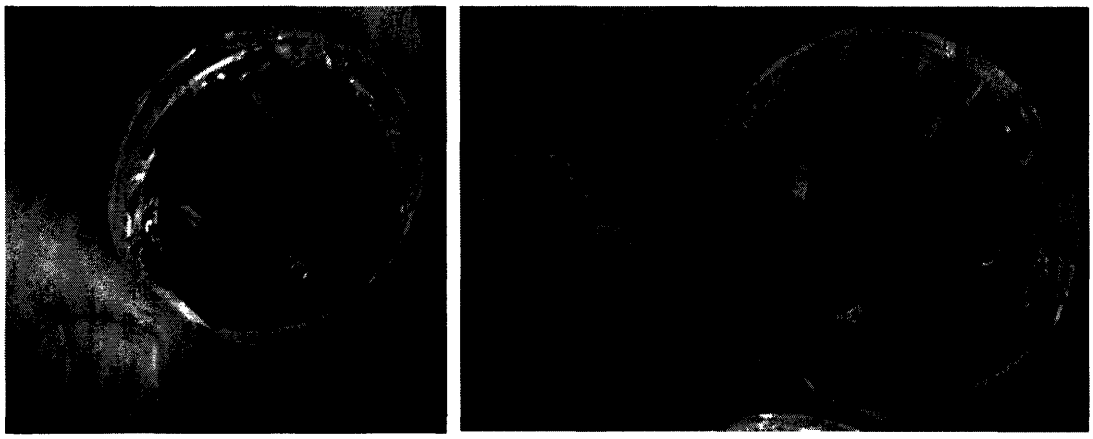
using 3D scaffolds contained in a polydimethylsiloxane (PDMS) micro fluidics bioreactor. With this system, the surface shear stress, as well as the pressure difference and biochemical gradient across the cell matrix can be controlled. Recent work has shown that endothelial cells grown in the bioreactor organize into capillary-like structures which suggest the initiation of the neovascularization processes.[6] To further study angiogenesis, smooth muscle cells must be incorporated into the bioreactor system. Thus, the goal of this thesis is to mimic the *in vivo* vascular remodeling function of smooth muscle cell migration in a 3D environment and develop the technology needed to view this growth in real-time.

Mesenchymal precursor cells, 10T1/2 cells, were grown in a three-dimensional collagen matrix. The three-dimensional bioreactor developed for endothelial cell growth by V. Vickerman was used for the present 10T1/2 cell experiments. Cell morphology and migration were observed in response to different doses of PDGF-BB. PDGF-BB was chosen as the growth factor to use because studies show that both smooth muscle precursor and smooth muscle cells are stimulated to migrate by endothelial cell media and specifically PDGF-BB. When the PDGF-BB action is blocked in 10T1/2 cells with neutralizing antibodies, the migration and proliferation increase that is observed with endothelial cell co-culture is not observed.[4] These studies were conducted *in vitro*, and similar results are found *in vivo*. Mice that are PDGF-B receptor deficient have leaky and abnormal vessel formation.[7] 10T1/2 cell proliferation and migration, as observed by others, increases in a dose-dependent manner, which is why this study uses multiple conditions to test the migration of the 10T1/2 cells.[4, 8]

The analysis of chemical gradients and penetration of signals in a unique three-dimensional environment is an important step in understanding neovascularization. A chemical gradient of PDGF-BB is set up across the 10T1/2 cell scaffold and migration is observed in real-time. Currently, the cells in the bioreactor are grown in an incubator and need to be removed for observation. To understand the full scope of smooth muscle cell proliferation and migration, the cells need to be viewed in real-time.

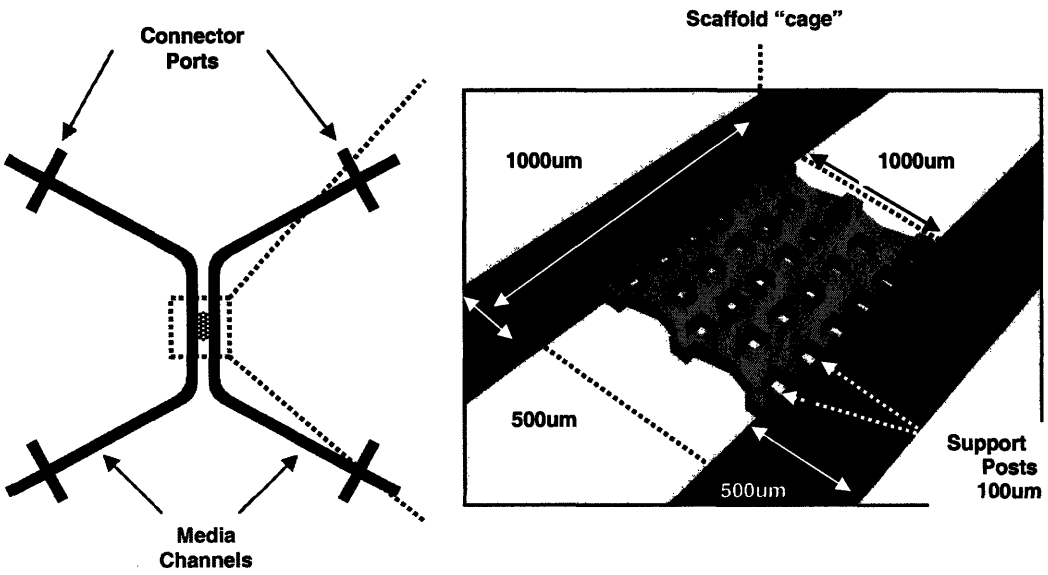
1.2 Bioreactor and Bench-top Incubator Device Design

The current bioreactor, made of PDMS gel, is pictured in Figure 2 and the bioreactor design is depicted in Figure 3. Cells are introduced either by being transported in the channels of the bioreactor (through the connector ports in Figure 3) or injected directly into the collagen before it has hardened.



A B

Figure 2: PDMS gel: channels are clearly visible in **A** and size comparison in **B**.



A B

Figure 3: Bioreactor design: **A)** Media channels and connector ports **B)** Larger scale view of collagen scaffold, black depicts media channels, gray - collagen scaffold, white - PDMS gel.[6]

After the cells have been placed in the collagen gel, the PDMS bioreactor is placed in a clamp. The clamp is depicted in Figure 4. The connector ports of the bioreactor are connected to media flow. The bioreactor and clamp is then placed in an incubator. The clamp was originally used as a way to hold the bioreactor in place while fluid was flowed through the device.

Figure 4: The bioreactor is placed in the clamp base (left) and the top (right) is inverted and clamped on top

Different growth factors can be circulated through the media channels in the system to achieve a chemical gradient in the collagen scaffold. Currently, in order to image cells at different time-points throughout an extended experiment, the device must be removed from the incubator and placed under a microscope. The cells cannot survive outside the incubator for long periods of time at room temperature and low humidity; therefore, observation time must be kept short. Because of this, the clamp was integrated into a custom-designed climate controlled incubator, so that the clamp can be kept on the bench-top and the cells can be viewed in real-time.

The incubator device includes heat coils, a temperature sensor, and a carbon dioxide transport system. This system makes it possible to grow smooth muscle cells and view them on the microscope stage. The effect of chemicals and environmental control changes can be seen instantaneously because of the ease of real-time viewing of the sample. Further

applications of the bench-top incubator include growing endothelial cells and smooth muscle cells simultaneously in the bioreactor. The interaction between the cells and the eventual capillary-like formation can be viewed continuously, providing a powerful tool for studying new vessel formation.

Such a bench-top vascular system has broader usefulness for pharmaceutical development and drug testing. Companies could theoretically be able to perform the first round of product testing on *in vitro* capillaries instead of solely using endothelial and smooth muscle cells in 2D. The changes to the cells due to the tested products could be observed immediately using the real-time viewing capabilities of the bench-top incubator device and would eliminate the need for animal models in the early stages of testing.

Chapter 2: Methods

Smooth muscle precursor (10T1/2) cells were used in the experiments. A migration assay for both 2D and 3D cell growth was developed and used to quantify the movement of cells in response to the addition of PDGF-BB. The cells were grown in both 2D and 3D. The results of the thesis focus on the cell growth in 3D, but the 2D growth was needed to understand and optimize the growth of the 10T1/2 cells. The details of the cell growth protocols and the experimental techniques used are noted below.

2.1 Cell Growth

10T1/2 cells (CCL-226, ATCC, Manassas VA) passages 9-11 were used in all experiments. When cultured for three days prior to device injection, the cells were grown on Collagen (50 ug/mL Type 1 collagen in 0.02N acetic acid) or Gelatin (0.1%) in DMEM growth media (Dulbecco's Modified Eagle Medium with 4.5 g/L D-Glucose, and L-glutamine, without Sodium Pyruvate (Invitrogen, Grand Island, NY), with 10 % Fetal Bovine Serum (Cambrex Bio Science, Walkersville, MD), and 0.5% Penicillin streptomyosin))

2.1.1 Passage Protocol

The media was aspirated and the cells were rinsed twice with 1 mL warmed PBS (Dulbecco's Phosphate Buffered Saline, 1X, without Calcium Chloride or Magnesium Chloride (Invitrogen, Grand Island, NY.)) The PBS was aspirated and 1 mL warm trypsin (0.05% trypsin- EDTA, 1X, Invitrogen, Grand Island, NY) was added. The flask was placed in the incubator for 1 min or until the cells had detached from the flask. 4 mL growth media was added and the mixture was centrifuged at 1000 RPM for 5 min or until a pellet formed. The media was aspirated and the pellet was resuspended in 12 mL growth media. 4 mL media with cells was transferred to each of the three newly coated culture flasks.

2.1.2 Culture Flask Coating

Collagen

1 mL Type I collagen solution (50 ug/mL Type 1 collagen in 0.02N acetic acid) was added to the cell culture flask and incubated for 2 hours. The excess collagen was aspirated, and the flask was rinsed twice with 1 mL warmed PBS without Calcium and Magnesium.

Gelatin

4 mL gelatin solution (0.1%) was added to the cell culture flask and incubated for 15 min at room temperature. The excess gelatin was aspirated, and the flask was rinsed twice with 1 mL warmed PBS without Calcium and Magnesium.

2.1.3 Freezing Protocol

The cells were grown from passage 5 to passage 10 and then frozen. The cells were thawed two days before each experiment. The cells were frozen after being passaged. Instead of resuspending the cells in regular growth media, the cells were suspended in 1 mL FBS with 5% DMSO and placed in cryogen tubes. The cells were kept in liquid nitrogen.

2.2 Fluorescence Staining

The cells were fluorescently stained before they were used in the devices. (PKH26 Red Fluorescence Cell Linker Mini-Kit, Sigma- Aldrich Inc., St. Louis MO)

The cells in the culture flask were rinsed in serum free media (Dulbecco's Modified Eagle Medium with 4.5 g/L D-Glucose, and L-glutamine, without Sodium Pyruvate (Invitrogen, Grand Island, NY) and with 0.5% Penicillin streptomyosin).

2 mL 2.5 x 10^6 M PKH26 Red Fluorescence Cell Linker (5µl of 1 x 10^6 M Pkh26 Linker in Ethanol and 1.995 mL Dilutant C) was added to the cells and incubated for 6 min at room temperature. 2 mL FBS was added and the cells were incubated for another minute. The cells were rinsed three times and resuspended with regular growth media.

The cells fluoresce for more than five days with minimal photo bleaching.

2.3 PDGF-BB

The PDGF-BB (recombinant mouse platelet-derived growth factor-BB (BioSourse International, Camarillo, CA)) was diluted into 5 ug/mL or 100 ug/mL aliquots and stored in

20 and 50 uL stock supplies until the day of the experiment. The aliquots were prepared by reconstituting 10 ug of PDGF-BB in 100 mM acetic acid (99% pure, Sigma- Aldrich inc., St. Louis MO)) and 0.1% BSA (Albumin, Bovine Serum Fraction V, Crystalline, CalbioChem)

The stock was diluted in growth media to concentrations of 5, 7.5, 10, and 50 ng/mL in 2% FCS growth media.

2.4 Migration Assay

The cells were plated in C6 plates at a concentration of 125,000 cells per well. They were grown over night or until confluent and scratched with a 1 mL pipette tip to create a gap in the cell layer. The gap was approximately 700 – 1100 um in width.

In one trial, 2% FBS growth media was added to three of the wells and 2% FBS growth media with 5ng/mL PDGF was added to the other three. The scratch width was reported at 5, 12 and 24 hrs, and the average migration per hour was calculated. The cells were rinsed twice with PBS with Calcium and Magnesium (BioWittaker, Walkersville MD) before the media was added.

In the second test, the above experiment was repeated with 7.5ng/mL PDGF, 10ng/mL PDGF, and regular 2% FBS growth media in two wells each. The results at 0 and 15 hours were compared.

2.5 Bioreactor Devices [9]

2.5.1 PDMS Preparation

The SYLGARD 184 Silicone Elastomer (Daw Corning, Midland, MI) was used to make the devices. The Base and Curing agent were mixed in a 10:1 ratio; approximately 100 mL was used for four PDMS devices. The agents were mixed together and placed in the vacuum dessicator to remove air bubbles for 45 min. The mixture was poured onto the silicone wafers and placed in the dessicator again for 45 min. The devices were cured overnight in an 80°C oven or for two days at room temperature.

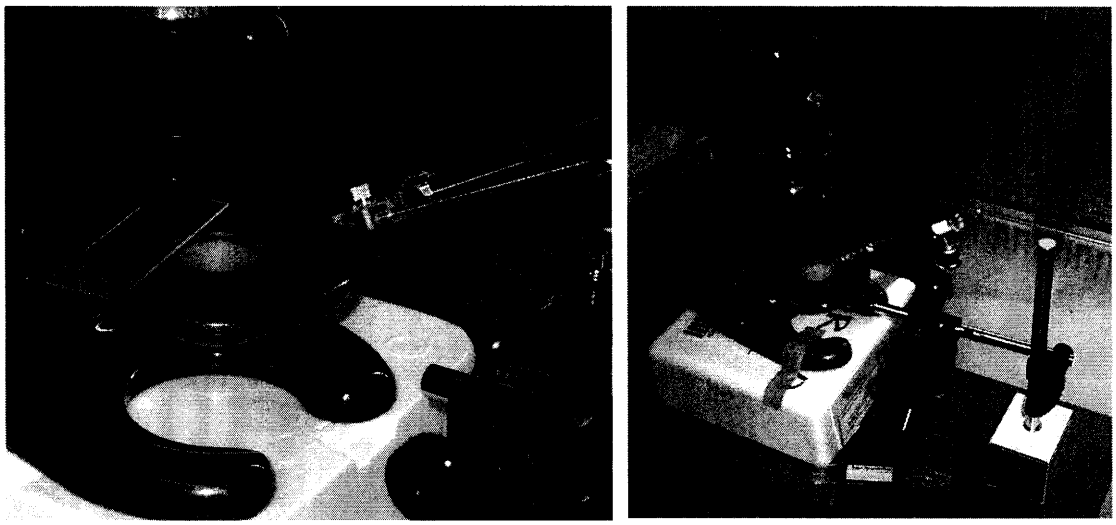
2.5.2 Device Preparation

The PDMS devices were placed in distilled water and run in the autoclave for a 20 min wet cycle. The devices were moved to a dry pipette tip box and autoclaved for 25 min

dry cycle, 15 min dry time. The devices were then plasma-sterilized four at a time for 2.5 min.

2.5.3 Cell Implantation

20 uL PBS (10X with Phenol Red), 3.6 uL 1 M NaOH, 115 uL Type 1 collagen (3.50 mg/mL, BD BioSciences, Bedford MA), and 30 uL distilled water were mixed with 32 uL media suspended strained cells to make the collagen-cell mixture right before the devices were injected. The cells are at a concentration of $2x10^6$ cells/mL in the media and the final concentration of cells in the collagen-cell mixture is $3.2x10^5$ cells/mL. 40 min after the plasma treatment, the devices were injected with a drop of the collagen-cell mixture. The injection set-up is seen in Figure 5



A B

Figure 5: Picture of needle and microscope: **A)** Microscope stage and needle **B)** Overall view.

Once injected, an autoclaved coverslip (35mm circular) was placed over the PDMS device and the device was placed in a hydration box (50 mL water in a 1 mL pipette tip box, autoclaved with the devices in the wet cycle). The collagen was cured for 40 min in the incubator, and 70 uL of growth media was placed on each channel and 20 uL was pulled through each channel. The media was exchanged in a similar fashion every 12 hrs during cell observation.

When testing the PDGF-BB, the regular growth media was replaced by 2% FBS growth media 12 hrs after injection, and replaced by 5, 7.5, or 10 ng/mL PDGF-BB in one of the channels 36 hrs after injection.

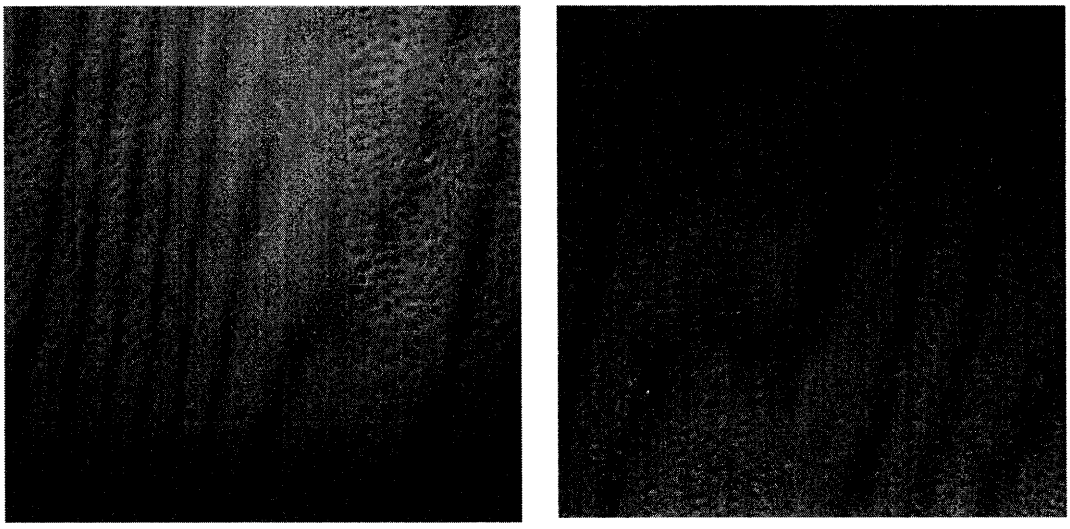
The devices were photographed at 4, 8, 20, 28, and 50 hrs. The images at 0, 28, and 50 hrs were superimposed and the distance traveled by the different cells was measured using the pixval function in Matlab. The distance between the centroid of the cells is measured in pixels and converted to um using the conversion 1mm:428 pixels in 4X and 1mm:1560 pixels in 10X.

Chapter 3: Results

3.1 Smooth muscle cell migration assay development

The goal here is to develop an assay for studying smooth muscle cell migration in response to a number of stimuli, including growth factor stimulation and endothelial-derived product. Initially application of PDGF-BB, a know stimulator of smooth muscle cell migration, was used to develop the assay. First, a scratch migration assay in 2D was conducted to test the effectiveness of PDGF-BB on the cells and determine what concentration should be used in the devices. Since the PDGF-BB is reconstituted in acetic acid, the higher concentrations of PDGF-BB may be toxic for the cells due to the higher concentration of acetic acid.

The migration speeds of cells in 5 ng/mL PDGF-BB in 2% FBS and the control cells in just 2% FBS media were compared. Pictures were taken at 0, 5, 12, and 24 hrs. The average speed of gap closing was calculated between 5 and 12 hours after the addition of PDGF-BB. This was done by comparing the gap size at different time points. An example of the gap size change is seen in Figure 6.



A B

Figure 6: Cells in 2% FBS growth media at **A)** 5 hrs and **B)** 12 hours

The average speed of the control was 52±9 um/hr and the average speed of the PDGF-BB exposed cells was 77±5 um/hr, showing the effectiveness of PDGF-BB at 5 ng/ml for stimulating 10T1/2 cell migration. Three trials were completed for each media and the results can be seen graphically in Figure 7. The difference in speed is statistically significant ($p<0.05$)

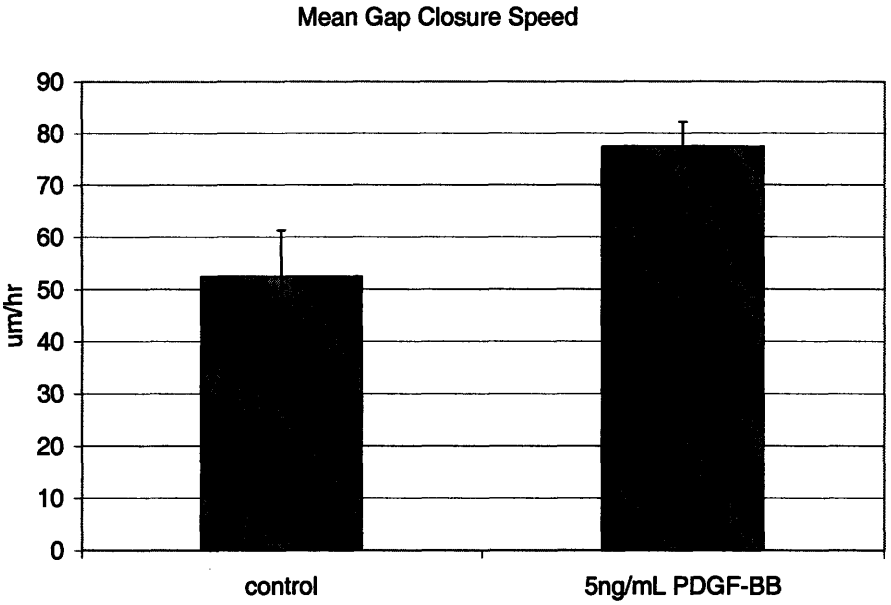


Figure 7: Average speed of gap closure from 5 hrs to 12hrs after cells were exposed to PDGF-BB

Once the efficacy of PDGF-BB as a stimulator of 10T1/2 cell migration in 2D was established, the growth factor was used to stimulate migration in the 3D bioreactor. First, an understanding of the growth factor gradient across the gel scaffold region must be achieved. The PDGF-BB concentration across the collagen scaffold reaches steady state after 3 hours. The diffusion coefficient of PDGF-BB in collagen is estimated to be $1x10^{-10}$ $m^2/sec.$[10] Using Equation 1; the time it takes to reach steady state is calculated.

$$t = \frac{l^2}{D} \qquad (1)$$

Where l is the width of the collagen scaffold, 1 mm, and D is the diffusion coefficient of PDGF-BB. Furthermore, the gradient of PGDF-BB throughout the collagen matrix is linear at steady state. The initial concentration of PDGF-BB in one channel and the

control media is the other are assumed to have an infinite supply, representing a constant flux at each boundary.

Next, the 3D collagen gel scaffold-cell mixture and the injection technique were optimized for smooth muscle cells since the original bioreactor and protocol were designed for endothelial cells. The optimization is discussed in Chapter 4.

Once the cell growth in 3D was successful, the 10T1/2 cells were stained with a fluorescent live cell tracker and imaged at 0, 4, 8, 21, 28, and 50 hours at 4X and 10X. The images from 0, 28, and 50 hours were superimposed to make the movement of the cells quantifiable.

An example of the difference between bright field and fluorescence along with the superposition of the fluorescence images from the different time points can be seen in Figure 8 for a control device containing no growth factor stimulation.

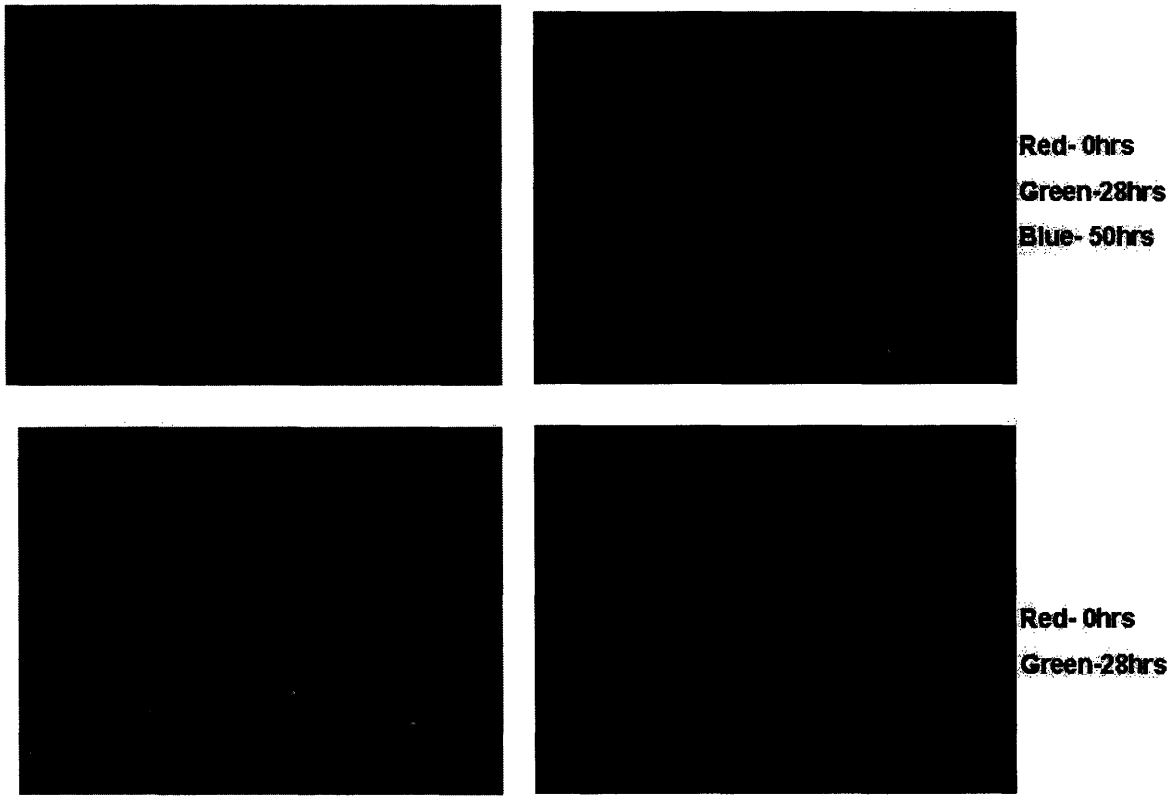


Figure 8: Bright field and fluorescent superposition of different time points of two different control devices (2%FBS growth media in both channels)

A similar set of images for the 5 ng/mL PDGF-BB in 2 % FBS growth media are shown in Figure 9.

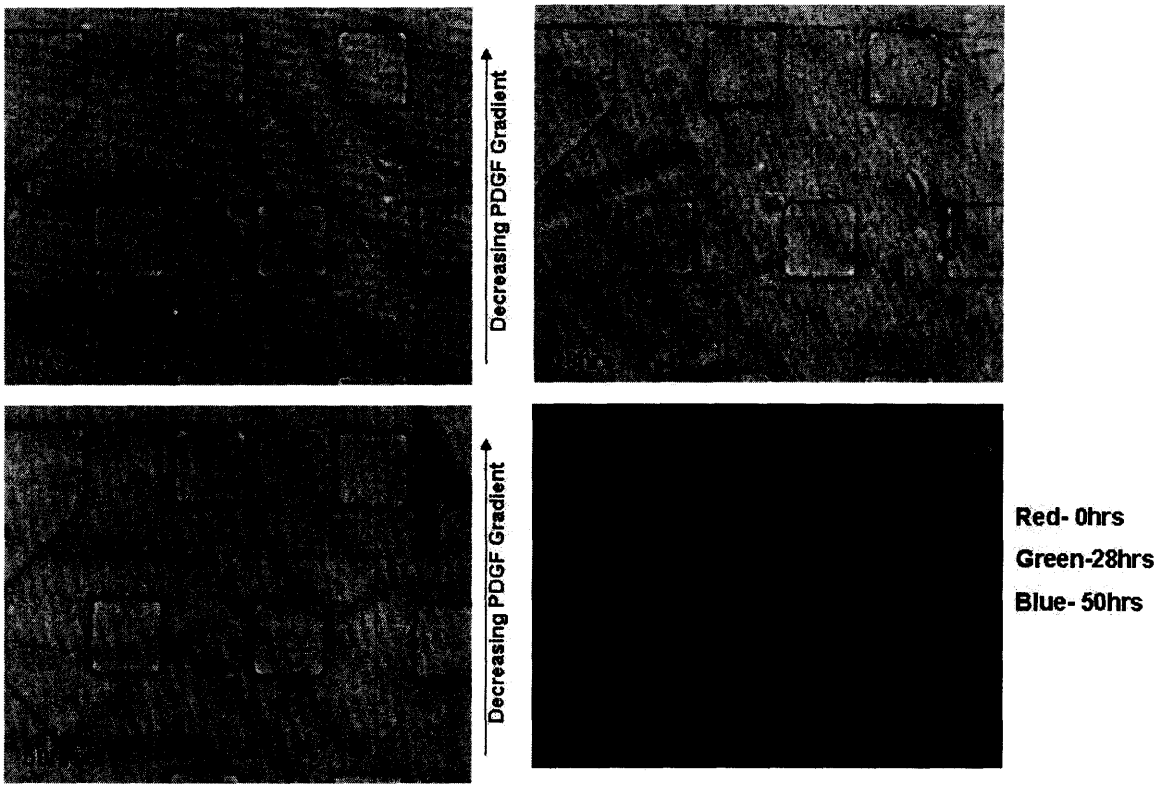


Figure 9: 5 ng/mL PDGF-BB in lower channel. 0, 28hrs, 50 hrs bright field images and fluorescence images superimposed.

Some of the cell movements were quantified from the 10X, but no conclusions could be drawn from the data. An example of cell movement can be seen in Figure 10. The PDGF-BB is applied on the bottom of the device, and the cells seem to be moving towards the PDGF-BB.

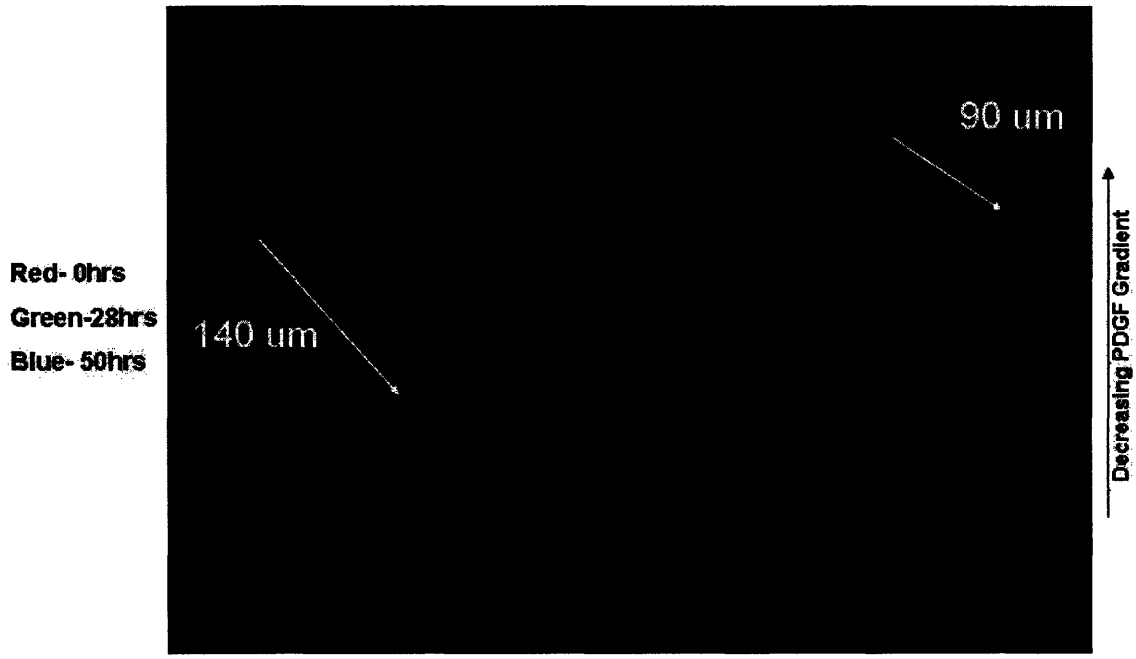


Figure 10: 7.5 ng/mL PDGF-BB in lower channel. 0, 28hrs, 50 hrs fluorescent images have been superimposed.

The marked cells have moved 140 um and 90 um. There were not many devices with such well defined movement as in this image; therefore, to quantify the movement of the cells, the fluorescent images of the devices in 4X were divided into a top and bottom section. At each time point, the amount of cells in each section was counted and normalized as a proportion to the total number of cells in the device. A picture of how the device was sectioned is seen in Figure 11.

Figure 11: Diagram of how the cells were counted

This was repeated for each concentration of cells at 0, 28, and 50 hrs. The data suggest cell movement towards the PDGF-BB. The cells in the control do not move while there is much movement in the devices with PDGF-BB. A diagram of the percent change in the half of the device closest to the PDGF-BB over 28 hours is seen in Figure 12. The PDGF-BB was applied to the bottom of the devices; hence, a change in cell proportion towards the 'Bottom' suggests the movement of cells towards the PDGF-BB.

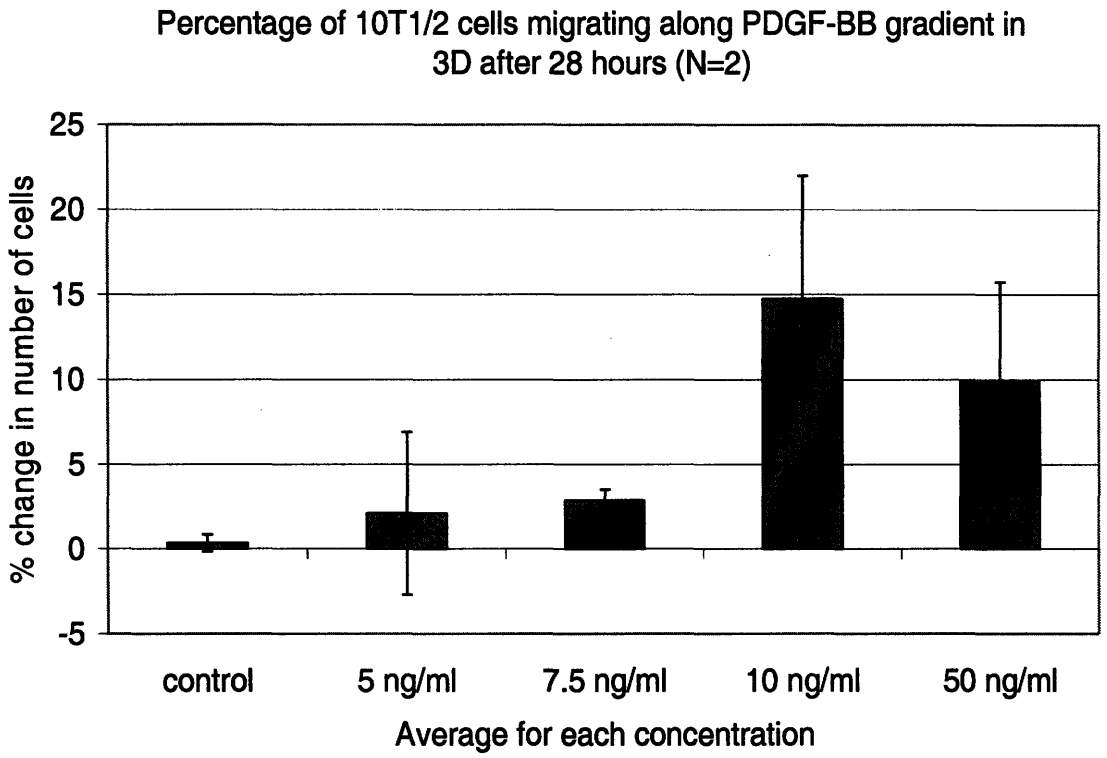


Figure 12: Percent increase with error bars in the number of cells in the section close to PDGF-BB over a span of 28hrs for 0, 5, 7.5, 10, and 50 ng/mL concentrations

The cells were also counted trying to capture the cell movement in the different areas of the PDGF-BB gradient. The cells were counted similarly to Figure 11, but each top and bottom section was split up in two portions. This way, the cell movement across four sections was analyzed. The graph of this data is seen in Figure 13.

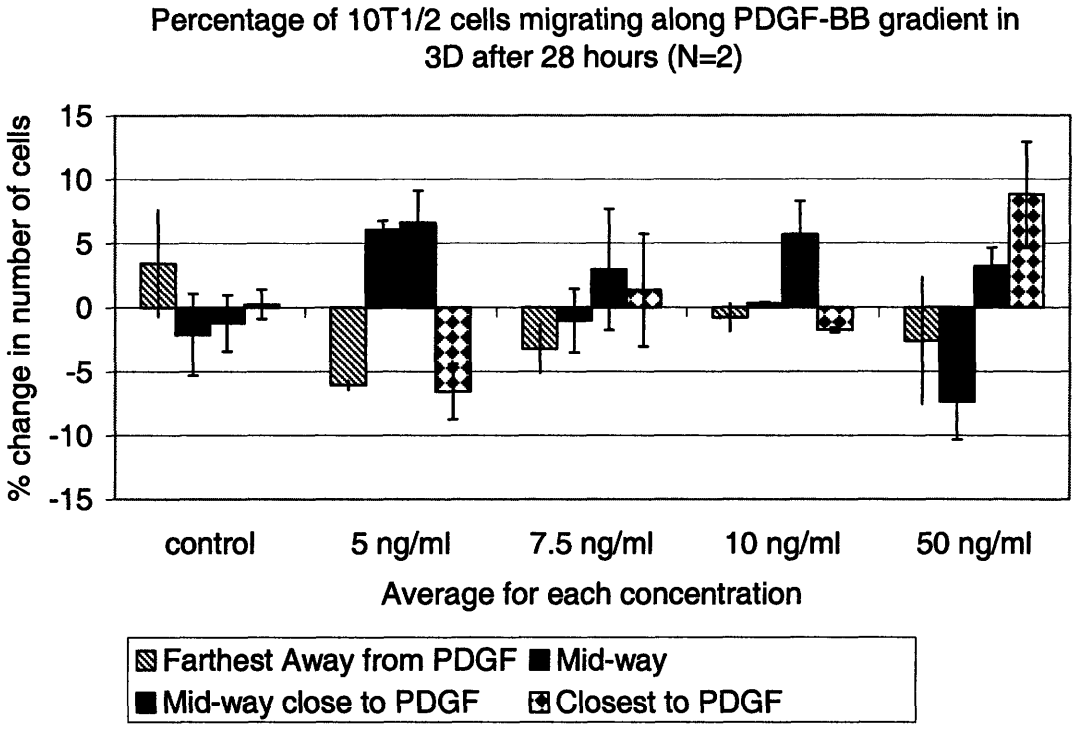
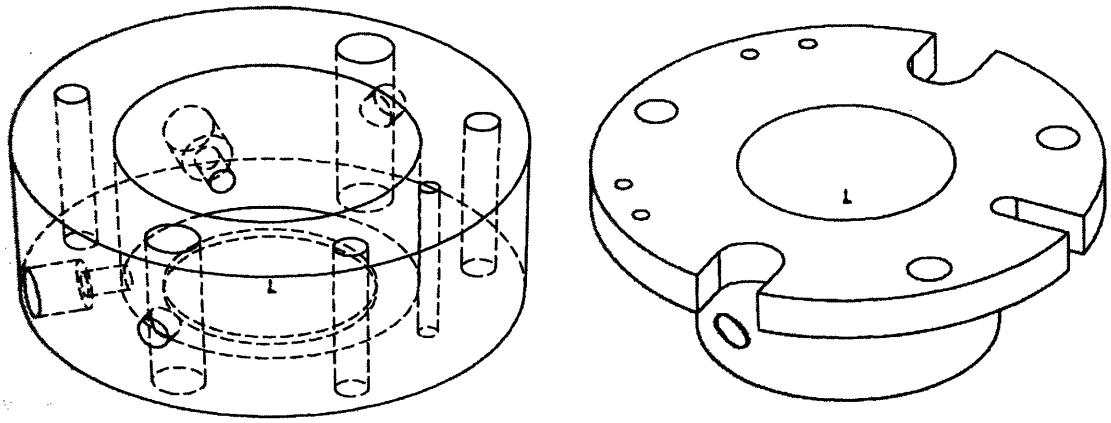


Figure 13: Percent increase in number of cells across the PDGF-BB gradient over a span of 28hrs for 0, 5, 7.5, 10, and 50 ng/mL concentrations

The distinct movement that would be expected is seen in the 50 ng/mL device. There are a decreased percentage of cells in the two top sections, and an increased percentage of cells in the sections closest to the PDGF-BB. The data is clearer and less variable in the initial assay analysis using two sections than in this analysis method.

3.2 Bioreactor Bench-Top Incubator Design

In order to create a bench-top incubator, multiple designs were considered. The current bioreactor clamp was chosen since the clamp was already designed to keep the bioreactors in place and did so successfully. Using this as the starting point, two heat coils (CIR Series, 120V length 2 in, 80 W, 1/4" diameter) were fit tightly on opposite sides of the device with the thermocouple (TP30 sensor) in between them. The temperature controller (5C1-140 Controller, Oven Industries, PA) was used to control the heat coils. The redesign of the top and bottom portions of the clamp is seen in Figure 14.



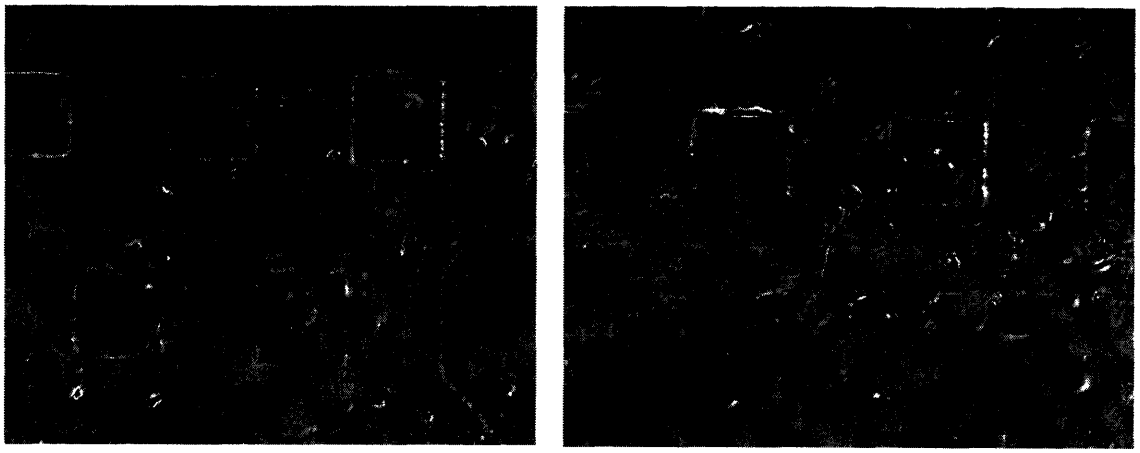
A B

Figure 14: A) bottom section of clamp with holes for heat coils (with screws to push the coils snug to the device), thermocouple, clamping mechanism, and carbon dioxide fittings. **B)** Top section of clamp with holes for the clamping mechanism and side hole for carbon dioxide flow above the PDMS. [11]

The temperature controller does not have a built in temperature sensor readout and required calibration in order to maintain the bioreactor temperature at 37°C. A temperature sensor was placed into a PDMS chip when it was poured, and the temperature was measured at the center of the chip when testing the device. It was allowed to equilibrate at 36°C and the thermocouple settings were kept constant for the cell tests. An Airgas mixture (5% CO_2 and 21% O_2 in a N_2 cell culture balance) was slowly flowed over the devices. The gas was bubbled through a heated water bath before entering the device to humidify the air. The final device with PDMS temperature probe is seen in Figure 15.

Figure 15: Bench-top incubator with temperature probe in PDMS along side the temperature controller.

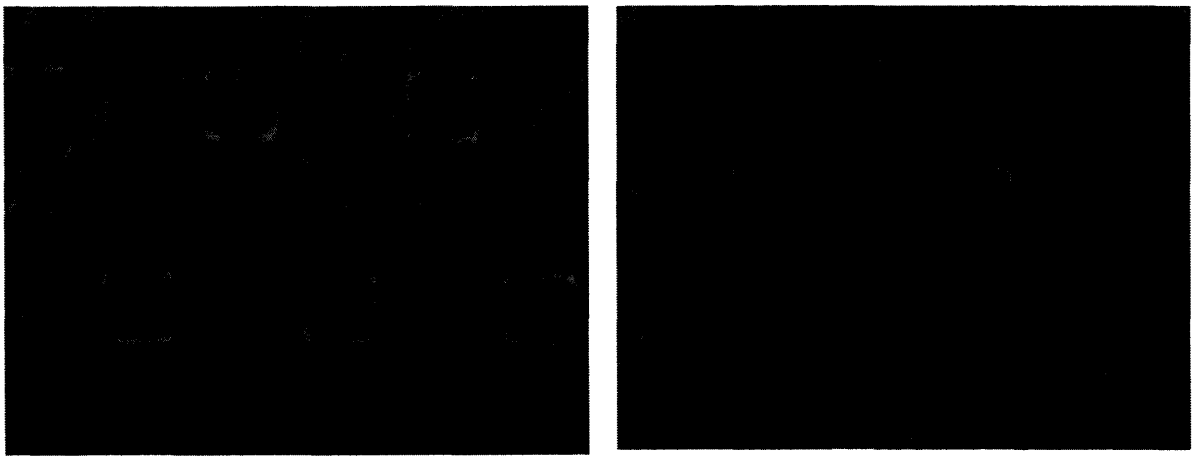
The PDMS test chip was replaced with a PDMS device with the collagen-cell scaffold. A control was placed on the bench-top, open to the environment and one in the regular incubator. The devices were inspected 12 hours post incubation. The cells in both the regular incubator and the clamp incubator survived and looked healthy, while the cells grown on the bench-top alone did not look healthy. Both the devices on the bench-top had dried out partially though. The one in the bench-top incubator had some fluid left in the ports while the device outside of the incubator had completely dried up. Figure 16 shows the cells from the device that was on the bench-top alone as well as an image of the drying out of the bench-top incubator device.



A B

Figure 16: A) Image of the device that was kept on the bench-top alone for 12hrs **B)** Picture of edge of the device after being in the bench-top incubator for 12 hrs.

A picture of the healthy cells before and after 12 hours of being in the bench-top incubator is seen in Figure 17.



A B

Figure 17: A) 64 hrs after injection **B)** 98 hrs after injection and after 12 hrs of being in the bench-top incubator.

The cells survived for 12 hours in the bench-top incubator and did not survive solely on the bench-top.

Chapter 4: Discussion

4.1 Smooth muscle cell migration in 3D

The cells grown in a PGDF-BB gradient migrated more than the cells grown in the controls. In general, the cells moved towards the PDGF-BB as seen in devices of all PDGF-BB concentrations. The most consistent results are seen in the 7.5 ng/mL devices. The cells in the 7.5 ng/mL devices show a marked migration towards the PDGF-BB with low variability. The cells grown in the 10 ng/mL gradient show more movement, but the movement is more variable. The 50 ng/mL tests were expected to show either lower variation in the data or more migration than the 10 ng/mL data, neither of which were found. When cells were plated in 2D and grown in 50 ng/mL PDGF-BB there was tremendous elongation and proliferation compared to the controls, however, the same result was not seen in 3D. Cells are known to migrate much slower in three dimensions. Therefore, the fact that the effect of PDGF-BB in 3D is not as great as in 2D is expected, yet a marked difference is predicted. In the 50 ng/mL device test, the humidity in the incubator was low and the devices dried out slightly over night. This could have attributed to the low migration of these cells.

The technique developed to be able to apply a concentration gradient to the cell scaffold is elaborate and many trials were conducted before cell survival was noted. There are many variables that if altered slightly effect cell survival.

The syringe diameter could potentially affect the cells when they are being injected into the device. The 2.5 uL syringe had an adequate survival rate of the cells passed through the syringe.

Devices with different collagen percentages and cell densities were made. The cells need to survive for over 96 hours and the collagen scaffold must be kept intact throughout the experiment. A low concentration of collagen led to the scaffold deteriorating before the end of the experiment while a high concentration led to higher cell death. 2% collagen, as noted in the methods section was chosen. Cell death in the devices was approximately 25-50%. When the devices had 20 or more cells, fluorescent imaging became easier and the variability between cells could be accounted for. If more than 50 cells were seeded per

device, the cells were difficult to distinguish, and the result of PDGF-BB addition could have been confounded by cell to cell contact. The cells were diluted to $3.2x10^5$ cells/mL in the collagen-cell mixture that was injected into the devices.

The cross linking of collagen in the hydration box seemed to vary between each experiment. Even with the same proportions of substances in the collagen mixture, the degree of cross linking would vary each time. This could be due to slight inadvertent variations in the protocol such as how long the collagen had been in the needle before injection or how much water was in the hydration box. These changes were harder to control or identify, but after much trial and error a consistent protocol was developed. Adhering to the details of the protocol is important for low variability of the results.

Bubbles in the needle or in the collagen were also an issue. If bubbles were trapped in between the PDMS and coverslip, the collagen scaffold would not be continuous in between the posts and would deteriorate quickly. To eliminate bubbles, the needle was rinsed multiple times until it was filled with bubble free water. The water was replaced with collagen, and rinsed again with the collagen-cell mixture. This eliminated the bubbles in the scaffold, and also ensured that there was no variability in the amount of potential water or air that the cells had come in contact with before being injected into the device. The swift placement of the coverslip over the PDMS following injection was also important. The coverslip was simply placed on the PDMS and allowed to adhere without applying pressure.

The small differences between each device such as the ones mentioned above can introduce variability between the samples. Any one of the changes could affect the microstructure and mechanical properties of the collagen and in turn influence the migration of the cells.

Another device design issue that has been addressed is that even though the cells survived and were observed for 90 hours, after 50 hours, the glass coverslip started to detach from the PDMS. In the detached devices, the PDGF-BB gradient could no longer be imposed, which could have led to the cells moving without a regular pattern. This could be due to many factors. Some potential factors were that the devices got too wet during the collagen cross linking step and detached from the coverslip. The hydration box was wiped

down before the devices were placed in the box to ensure that the device did not come in direct contact with water.

The Plasma Sterilizer used to plasma treat the devices had no quantifiable way of measuring how hydrophilic the devices were. The amount of time that the devices were being plasma sterilized was varied between 2 and 3 minutes, 2.5 min being the most effective time. If the cells were injected too soon after the treatment, the collagen would not stay in the scaffold areas but would spread out into the channels. If the injection happened too late, the PDMS would no longer be hydrophilic and the collagen would form a drop on top of the PDMS and not spread out and into the scaffold cage. Even with consistent times in the plasma treatment, the required waiting time before injection varied. 40 minutes was approximately the appropriate time, but some devices were not the correct hydrophilicity after 40 min. The variations in hydrophilicity could be dependant on the placement of the devices in the plasma treater. No direct correlation between location and treatment strength could be made though. As a way to try to reduce leakage, the coverslip was also plasma sterilized. The devices that used these coverslips had patchy scaffolds, indicating that the collagen had spread out from the scaffold cage area into either the channels or in between the PDMS and the coverslip.

If there was an easy way to measure the hydrophilicity of the PDMS without injecting collagen into it, all the cells could be injected using the same conditions and the variability between devices may decrease.

Having a solid bond between the coverslip and PDMS is important in being able to keep the concentration gradient and avoid leakage. The cells grown in 2D seemed to migrate more quickly at least 18 hours after PDGF-BB addition. Since cells migrate slower in 3D, longer time periods are necessary for 3D movement. If leakage could be eliminated, the cells could be viewed for longer periods of time and more marked migration patterns may be observed.

The 3D migration assay that was developed is a good way to observe general cell movement. As seen in Figure 10, trying to observe movement in 10X is difficult due to the multiple layers of cells in the 3D scaffold. There are four or five layers of cells and

fluorescent images would need to be taken of each layer to see the full cell movement. The amount of data that would be collected is far too large to analyze quickly.

In the 10 X images, some cells move out of sight during a series of pictures because they move vertically in the scaffold or out of the image region. Also, if the cell density is too high, it is hard to differentiate between cells and know which cells migrate where. Because of this an assay that would quantify the general migration patterns of the cells was developed.

By using a lower magnification and not requiring the tracking of individual cells, the trends of the cells can be noted. When comparing the results of the two assays, the first one, measuring the migration over the center boundary, shows more concrete results. When viewing the migration split up into sections, the effect is not as well defined, and the variation in movement is large. The second assay would yield better results if a higher seeding density was used. In this experiment, due to the lower cell density, the variability of the cells and human error in counting outweighed the potential benefits of being able to see the differences in cell movement throughout the concentration gradient. The data is promising though, and there seems to be a threshold concentration between 7.5 and 10 ng/mL. In future research, a minimum of 10 ng/mL PDGF-BB should be used to note a change in migration of smooth muscle precursor cells in 3D.

Both assays can be used to note the migration of cells in many other chemical gradients such as different growth factors, conditioned media, or endothelial co-culture.

Improvements can be made upon the experiments conducted. The first and foremost being repeating the experiments. Larger sample populations are needed to make clear statements on the effect of PDGF-BB on smooth muscle precursor cells in 3D. Each concentration has only been tested two or three times, making none of the results in 3D statistically significant. The trends of the cells suggest that if more trials were completed, significance would be found.

4.2 Bioreactor Bench-Top Incubator Design

The bench-top incubator kept the 10T1/2 cells alive for an extended period of time at 36°C. The humidity in the device was not kept high enough though, and the bioreactor dried out in the bench-top incubator more than in a regular incubator. This could be solved by encapsulating the bench-top incubator in a clear box with water reservoirs inside. This would keep the whole volume at a constant humidity and still maintain the real-time viewing capabilities of the incubator.

The size of the incubator was taken into consideration when developing the design. Ideally multiple incubators would be on the microscope stage at the same time and would be rotated around to take pictures of all devices consecutively. Encapsulation of the incubator would hinder the ease with which this could be done.

The flow of the cell culture gas mixture was not kept constant throughout the trial period of the incubator. The tank regulator flow was dependent on the amount of gas left in the tank; hence, a flow regulator is needed to control the flow. This could also help in keeping the humidity high. The gas is bubbled through a water bath before entering the incubator; if the flow rate was slow enough, the flow would heat and humidify the system instead of drying it out which probably happened during the trial.

A series of valves would allow us to regulate the flow to a low enough rate that the volume around the device would not be displaced in less than 10 min. At this rate, the gasses would diffuse through the PDMS, but not remove the water from the device.

The temperature controller used for the incubator does not have a temperature readout, but has to be calibrated with a thermometer before the devices are placed in the incubator. If the temperature varies during the experiment there would be no way of noticing. A controller with a temperature readout is much more expensive than the current one. The temperature readout capabilities are probably not worth the increase in price since it would make it more difficult to make multiple incubators that run simultaneously. Having a temperature readout would give us more control and make the cell environment less variable though.

The basic requirements of the incubator were met and the cells survived long enough to note the benefits of real-time viewing.

Conclusion:

Many studies have been conducted on the migration, proliferation, and differentiation of cells in 2D. Using the tissue engineering platform of this experiment and the migration assay developed, the same studies can now be conducted in three dimensions, bringing the research one step closer to mimicking *in vivo* function.

Smooth muscle cell research in particular can lead to the understanding of the later steps of neovascularization. The smooth muscle precursor cells used in this experiment migrated towards the PDGF-BB in the 3D scaffold. Experiments that would observe the cells for longer time periods as well as experiments with more devices would probably lead to statistically significant results.

Incorporating smooth muscle cells with endothelial cells in a co-culture in the device would be very interesting. Observing smooth muscle precursor cells and endothelial cells together can be done in real-time and in three dimensions when using the combined bioreactor and bench-top incubator. The movement of the smooth muscle cells in response to endothelial cells could be compared to the smooth muscle cell migration in response to other growth factors or chemicals known to be involved in neovascularization. The cell co-culture could be observed continuously using the bench-top incubator and the cell to cell interactions could be recorded. The technique developed in this thesis is very promising as a stepping stone to further research in mimicking the *in vivo* functions of the cells involved in neovascularization.

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