The Influence of Fibronectin, Collagen, FGF and VEGF on Proliferation, Migration and Integrin Expression of Human Foreskin Fibroblast Cells

(Compared to Human Endothelial Cells)

Zeel Amin • Aimee Flores • Prathyusha Garimidi • Margarita Martinez • Viet Nhan • Adam Northrup • Melissa McCoy • Tawil, B, PhD• Biomedical Engineering

California State University Channel Islands • One University Drive • Camarillo, CA 93012

Channel Islands
CALIFORNIA STATE UNIVERSITY
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Abstract

Engineered biomaterials may be useful for enhancing wound healing and preventing bleeding in some patients. Human Foreskin Fibroblast 1 (HFF-1) secretes extracellular matrix components such as fibronectin, collagen and other proteoglycans. There is research indicating that a combination of HFF-1 cells and Human Umbilical Vein Endothelial Cells (HUVECs) may induce vessel formation. The effects of different ECMs on HFF-1 2D proliferation was investigated and highest proliferation was observed using fibronectin 10 µg/ml ECM rather than collagen 5 and 10 µg/ml or fibronectin 5 µg/ml ECMs. The effects of bFGF and VEGF (1,5,10 ng/ml) and fibronectin 10 µg/ml ECM on HFF-1 and HUVEC 2D proliferation was studied and highest proliferation was observed in both cells when grown in media with serum instead of with bFGF and VEGF in basal media. HFF-1 cells and HUVECs were grown in fibrin constructs made from fibrinogen (20, 40 mg/ml) and thrombin (20 U/ml) and maximum proliferation was seen using fibrinogen 20 mg/ml. HFF-1 cell migration was investigated by conducting a Boyden Chamber assay with varying concentrations of bFGF (0, 1, 5, 7 ng/ml) and it was observed that increasing bFGF concentration caused an increase in cell migration. Flow cytometry analysis demonstrated an increase in α_5 and β_1 integrin expression in HFF-1 when grown in 3D. It was also demonstrated in HUVECs grown in 3D that α_5 and β_1 integrin expression either decreased or remained the same respectively. An immunocytochemistry experiment showed HFF-1 secreation of a fibronectin network. Vessel formation was investigated via a HFF-1 and HUVEC 3D co-culture bead experiment.

Introduction

Biomaterials clinical uses have extended significantly for a wide variety of indications including burns, acute and chronic wounds, skin loss, surgical wounds, and dermatological diseases. After injury, the wound space is filled with a fibrin clot containing growth factors released by platelets and monocytes. Fibroblast migration from the periwound collagenous stroma into the fibrin-laden wound is critical for granulation tissue formation and subsequent healing. Fibronectin may be an essential component of the invasion process *in vivo* since fibronectin can bind to cells and to other extracellular matrix (ECM) proteins simultaneously. Fibrin gels, prepared from fibrinogen and thrombin (the essential proteins involved in blood clotting), were among the first biomaterials used to prevent bleeding and promote wound healing. Cell proliferation depends upon the different environmental conditions the cells are placed in. When combined together, fibronectin and collagen are involved in processes such as cell adhesion and morphology. Additionally, fibronectin and collagen contain binding sites for ECM components. Fibronectin is known to stimulate cell growth and migration, but research does not provide a complete picture of all the mechanisms involved. Considering that fibroblast proliferation and migration are key in the wound healing process, the aim of this study is to observe and compare Human Foreskin Fibroblasts cells (HFF-1) proliferation on fibronectin 10µg/ml ECM when treated with different growth factors at different concentrations (2D) and when suspended in a fibrin construct (3D). Research indicates vessel formation when Human Umbilical Vein Endothelial Cells (HUVECs) is co-cultured with HFF-1 cells, therefore HUVEC proliferation (2D, 3D) was conducted and compared to HFF-1 cell experiments. FACS analysis was conducted to detect the α_5 and β_1 integrin expression of HFF-1 cells in 2D and 3D and HUVECs in 3D. A Boyden Chamber experiment was conducted to investigate HFF-1 cell migration with bFGF. Lastly, a 3D co-culture of HFF-1 cells and HUVECs with Cytodex beads was performed to investigate vessel formation.

Materials and Methods

Cell Culture: Cell culture flasks were taken out of 37˚C incubation and 5% CO_2. Media was removed, cells were washed twice with PBS (5 ml), trypsin (5ml) was added, and cells incubated in 37˚C and 5% CO_2 for 15 minutes. Media was added (5 ml) and cells were transferred to a conical tube and centrifuged at 200 G for 5 minutes. After discarding the supernatant, the pellet was re-suspended in 1 ml of media, and 20 µl sample was inserted into cell counting slide to acquire cell concentration. ***2D Cell Proliferation:*** HFF-1 were seeded (10,000 cells per well) on 3 24-well plates coated with 5 µg/ml collagen, 10 µg/ml collagen, 5 µg/ml fibronectin, 10 µg/ml fibronectin; HFF-1 and HUVECs were also seeded (10,000 cells per well) on 3 24-well plate coated with 10 µg/ml fibronectin per cell line and treated with bFGF/VEGF (1,5,10 µg/ml), basal media, and media with serum. Media (0.5 ml) was added to each well and experiment was stored at 37˚C and 5% CO_2 until analysis (Day 0, 3/4, 6/7). For analysis, plates were washed with PBS (0.5 ml) and calcein (150 µl) was added. After a 15-minute incubation in the dark, the plate was inserted into a Filter Max F5 Multimode Microplate reader and micrographs were obtained using Olympus IX71 fluorescent microscope. ***3D Cell Proliferation:*** HFF-1 and HUVECs were suspended (10,000 cells per well) in fibrinogen (20, 40 mg/ml). The fibrinogen (150 µL) was evenly added to each well before thrombin (150 µL) was added. The plate was tilted from side to side before adding thrombin to the next well. Fibrin constructs incubated at room temperature before adding media (0.5 ml) and incubating 37˚C and 5% CO_2 until analysis (Day 0, 3/4, 6/7). Plates were washed with PBS (0.5 ml) and incubated in the dark with calcein (150 µL). Fluorescent micrographs were obtained and read in a Filter Max F5 Multimode Microplate reader. ***Boyden Chamber:*** 40 ml of cell solution was re-suspended in 8 ml of media. 600 µl of corresponding bFGF solution was added to each well, the chamber was placed back on bFGF-filled wells, 200 µl cell solution was added to each chamber, the plates were incubated at 37˚until analysis (Day 0 plates were incubated for 1 hour). Then, 20 µl samples were taken from the top (chamber) and bottom (well) and added to cell counting slides to obtain cell concentration. For Day 4 and Day 7, the media was removed from wells and inside the chamber, 600 µl of trypsin was added to the wells, the Boyden chambers were added back to the wells, 200 µl of trypsin was added to each Boyden chamber, 10 minutes was allowed for incubation, samples were placed on slides and counted via the Nexcelom Bioscience Cellometer Auto T4. ***FACS:*** HFF-1 incubated with an antibody (α_5, β_1) for 1 hour on ice, and shaken every 15 minutes for 1 hour. Samples were placed into FACS machine for analysis. For Day 7 2D proliferation, the media was removed and the wells were washed with 500 µl of PBS. The cells were trypsinized with 300 µl, incubated at 37˚C, and neutralized with 200 µl of media growth serum. After centrifuging at 400 G for 5 minutes. The media was removed from all tubes and the pellet was re-suspended with 350 µl PBS. The pellet was re-suspended with 250 µl of PBS. 2.5 µl of each antibody were added to separate Eppendorf tubes and incubated for 1 hour on ice before performing FACS. For Day 7 3D proliferation, fibrin constructs were vortexed and trypsinized to liberate cells before proceeding with established FACS analysis procedure. ***ICC:*** PFA was added to HFF-1 (seeded on a 24-well plate), cells were washed with PBS before 2% serum (PBST) was added. After a 45-minute incubation time at room temperature, cells were washed twice and primary and secondary antibodies were added. ***HFF-1 and HUVEC 3D Co-Culture:*** Cytodex beads were washed with PBS and placed in cell culture flasks. HFF-1 and HUVEC were added separately to beads in their respective media. Beads and cells were shaken for 2 minutes every 15 minutes within 2 hours and were placed in 37˚C incubation and 5% CO_2. Cells were washed with PBS, centrifuged, and diluted with media. On a 24-well plate, thrombin 20 U/ml (150 µL) was added to fibrinogen 20 mg/ml (150 µL). Cell seeded beads were added immediately on top. The plate incubated at room temperature for 30 minutes before adding another fibrin layer. After 1 hour, cells and/or media was added. Media was replenished at Day 3 and Day 7.

Results

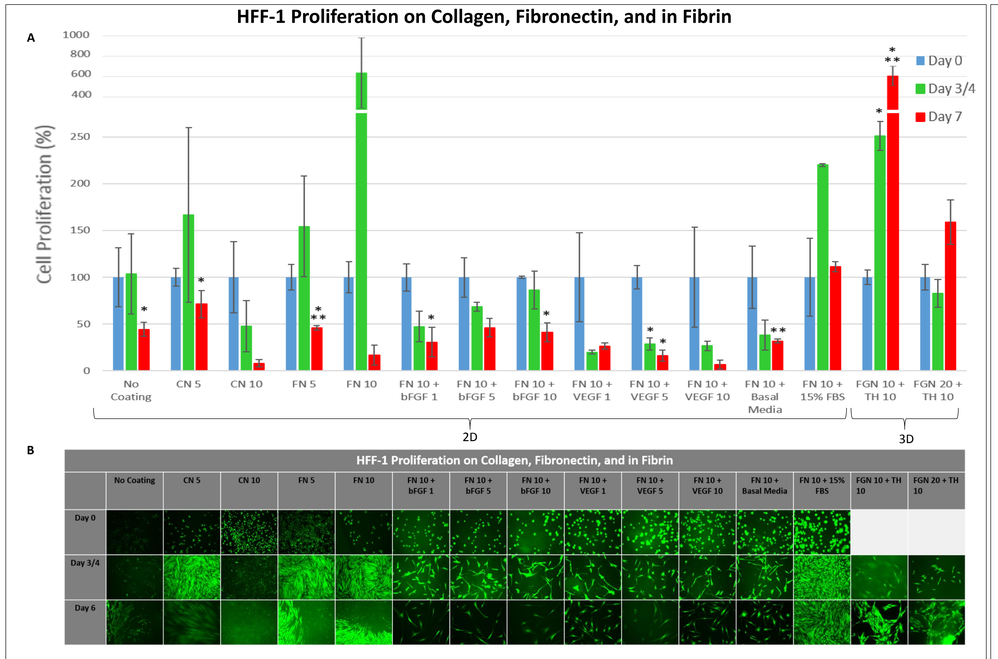

Figure 1: HFF-1 Proliferation on Collagen, Fibronectin, and in Fibrin: *Cultured HFF-1 cells in various conditions and labeled with calcein fluorophore in order to observe possible effects on proliferation and morphology.* ***(A)*** *Proliferative growth as measured at Day 0, Day 3 or 4, and Day 7 by the Filtermax F5 microplate reader, starting with 10k cells per well (n=3). Day 0 data was taken to be 100% and subsequent results were taken as a percentage of Day 0. Significance ($p < 0.05$) was calculated using t-tests between Days 0 and 3 (*), Days 0 and 7 (*), and Days 3 and 7 (**). HFF-1 cells were grown on different types and concentrations of matrix proteins (none, collagen, and fibronectin).* ***(B)*** *Using the IX71 fluorescent microscope with the QC Imager camera, fluorescent micrographs of HFF-1 cells in various conditions including 2D growth on collagen and fibronectin, growth in bFGF and VEGF on fibronectin, and 3D growth in fibrin clots.* ***Results: 1.*** *HFF-1 cells grown on different matrix molecules showed that fibronectin 10 ng/ml is the best when looking at Day 3 data ($p > 0.05$), though Day 7 cells decreased for all conditions ($p > 0.05$ for most conditions).* ***2.*** *Micrographs of this show that at Day 3/4 and 7, HFF-1 cells are spread and thus likely have formed attachments to their matrix.* ***3.*** *HFF-1 cells grown on 10 ng/ml fibronectin in various concentrations of growth factors showed decreases in cell numbers for all conditions including those in basal media, bFGF, and VEGF at Day 3 ($p < 0.05$ for most conditions) and Day 7 ($p > 0.05$ for most conditions). Cells grown in 15% FBS were the only ones to increase over time ($p < 0.05$).* ***4.*** *Micrographs of these conditions again show cell spreading and decreased numbers at Day 3 and Day 7 in comparison to Day 0. Cells grown in 15% FBS show substantially increased numbers at Day 3 and slightly increased numbers for Day 7.* ***5.*** *HFF-1 cells grown in fibrin clot show drastically increased numbers at Day 3 over Day 0 ($p > 0.05$) and at Day 7 over Day 0 ($p > 0.05$) and over Day 3 ($p > 0.05$) with 10 mg/ml fibrinogen. Cells grown in the 20 mg/ml fibrinogen fibrin clot increased overall at Day 7 ($p < 0.05$) but not nearly as much as the previous condition.* ***6.*** *Micrographs of these conditions show cell spreading for Day 3 and Day 7, and cells both in and out of focus display the different layers of cells within the clot.*

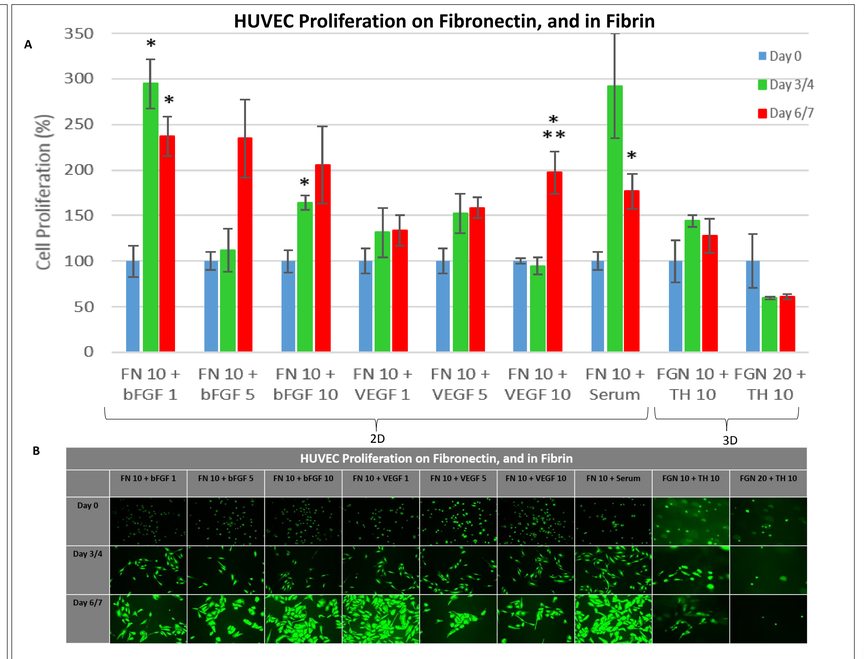

Figure 2: HUVEC Proliferation on Fibronectin and in Fibrin: *HUVEC cells grown in various conditions and labeled with calcein in order to study any effects on proliferation and morphology.* ***(A)*** *Effects on proliferation were observed at Day 0, Day 3 or 4, and Day 7 and data was recorded using the Filtermax F5 microplate reader (n=3). All conditions began seeded with 10k cells and day 0 was taken as 100%. Significance was tested using a t-test ($p < 0.05$) between Days 0 and 3 (*), Days 0 and 7 (*), and Days 3 and 7 (**). HUVEC cells were grown on 10 ng/ml fibronectin in HUVEC serum containing growth factors and in the presence of single additional growth factors (bFGF and VEGF).* ***(B)*** *Fluorescent micrographs of HUVEC cells grown in fibronectin, bFGF, VEGF, and fibrin clot.* ***Results: 1.*** *Cultures on various concentrations of bFGF and VEGF on 10 ng/ml of fibronectin show increased HUVECs at Day 3 and Day 7 in a similar pattern for most conditions ($p < 0.05$ for some conditions), but conditions reflect the same Day 3 and Day 7 quantities as those grown in serum ($p > 0.05$ at day 7).* ***2.*** *Micrographs show that HUVECs have spread at Day 3 and Day 7 over those cells at Day 0, though not as much as HFF-1s over the same period in Figure 1B.* ***3.*** *HUVECs grown on fibrin clots did not increase or decrease in number ($p > 0.5$ as expected), though those grown in 10 mg/ml fibrinogen may have shown a slight increase over time, which is in contrast to a slight decrease in those grown in fibrinogen 20 mg/ml.* ***4.*** *Micrographs of these conditions show only small spreading of those HUVECs grown in 10 mg/ml fibrinogen and no spreading in those in 20 mg/ml fibrinogen.*

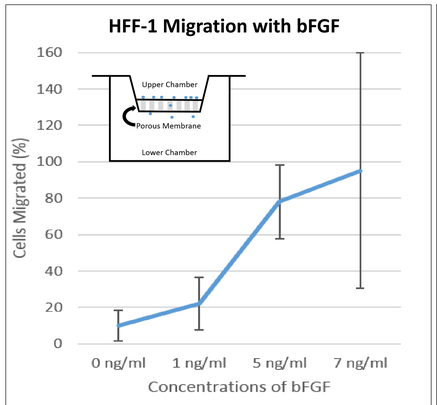

Figure 3: Migration of HFF-1 cells using Boyden chamber: *5,000 HFF-1 cells were added to the top chamber and quantified using a cellometer. As a result of chemotaxis, a unidirectional flow was established and the cells migrated through the porous membrane (0.8µm) to the lower compartment and cells were quantified using a cellometer after Day 4.* ***Results: 1.*** *Increased migration rate of HFF-1 cells is associated with increase in concentration of bFGF over 4 day period. About 2 fold increase in migration rate of HFF-1 cells was observed in 1 ng/ml bFGF on day 4 compared to 0 ng/ml of bFGF.* ***2.*** *An 8-fold migration rate increase in 5 ng/ml bFGF and a 9-fold migration rate increase in 7 ng/ml bFGF was observed when compared to HFF-1 migration rate in 0 ng/ml bFGF on Day 4.*

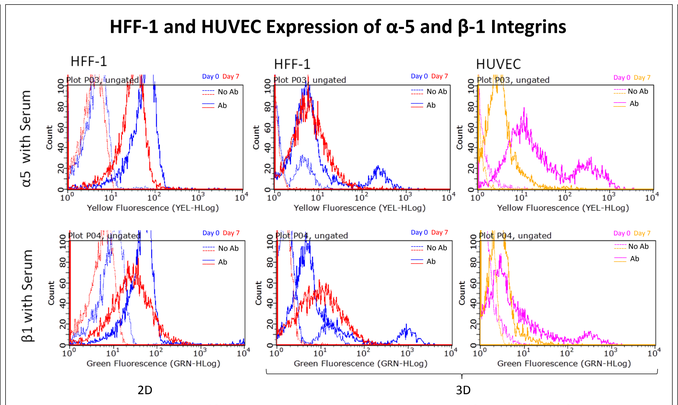

Figure 4: HFF-1 and HUVEC Expression of α_5 and β_1 integrins: *FACS analysis was conducted to investigate α_5 and β_1 integrin expression of HFF-1 cells grown in 2D and 3D proliferation environments in comparison to expression in HUVECs grown in a 3D proliferation environment. Expression of α_5 and β_1 integrins indicates cell migration and adhesion.* ***Results: 1.*** *By Day 7, a decrease in α_5 and β_1 integrin expression was observed in the HFF-1 cells grown in 2D (n=2) as indicated by a shifts to the left of the Day 0 curve.* ***2.*** *On Day 7, an increase in both α_5 and β_1 integrin expression was observed in HFF-1 cells grown in 3D (n=2) as indicated by shifts to the right of the Day 0 curve.* ***3.*** *When compared to HFF-1 cells grown in 2D, HFF-1 cells grown in 3D show more α_5 and β_1 integrin expression by Day 7.* ***4.*** *For HUVECs grown in 3D (n=2), less α_5 integrin expression was observed on Day 7. Additionally, approximately the same amount of β_1 integrins were expressed on Day 7 as Day 0.*

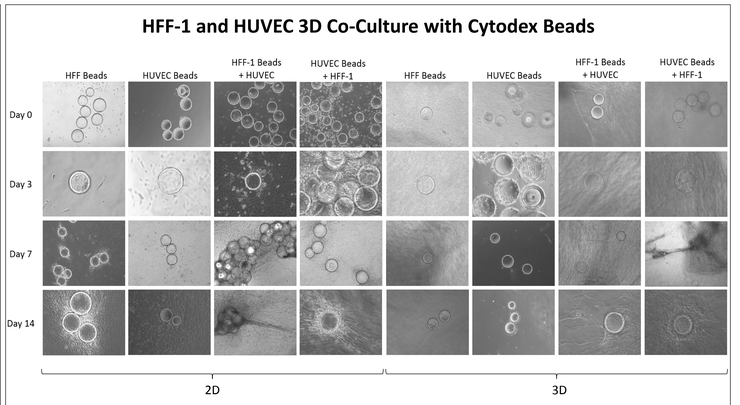

Figure 5: HFF-1 and HUVEC 3D Co-Culture with Cytodex Beads: *Vessel formation was investigated by adding HFF-1 cells and HUVECs with and without beads to 3D constructs made from fibrin. Cytodex beads were seeded with either HFF-1 or HUVEC and were placed in between layers of fibrinogen 20 mg/ml polymerized with thrombin 20 U/ml. HUVECs (20,000 cells per well) were added to HFF-1 seeded beads layered between fibrin and HFF-1 were added to the HUVEC-seeded beads. HFF-1 and HUVEC media were added to HFF-1 and HUVEC-seeded bead conditions respectively as negative controls. Similar conditions were also done in 2D. The beads were monitored over a period of two weeks, replenishing the media at Day 3 and Day 7 time points.* ***Results: 1.*** *Both 2D and 3D cultures with HFF-1-seeded beads and HUVECs show the formation of fibrous structures extending away from beads and clumps of cells starting at Day 7 and Day 14.* ***2.*** *Cells in 3D co-culture conditions may have shown more robust fibers extending away from beads and more clumping of cells both near and away from beads than 2D co-culture conditions.* ***3.*** *Cells were shown to be mostly located around beads for all conditions, but in 3D conditions, cells formed clusters nearby but autonomous from beads (not shown).* ***4.*** *Cultures with only one cell type did not show extensions from the HFF-1 or HUVEC cells.*

Discussion

- **2D HFF-1 Proliferation: 1.** Maximum HFF-1 proliferation was observed on fibronectin 10 µg/mL coating when compared to uncoated, collagen 5 µg/ml, collagen 10 µg/ml, and fibronectin 5 µg/ml extracellular matrix conditions. This may be because the fibronectin allows for greater attachment of HFF-1 cells, allowing for more intercellular connection thus increasing proliferation. **2.** Therefore, we chose fibronectin 10 µg/ml ECM in further experiments. **3.** HFF-1 cells grown with one growth factor demonstrated low proliferation. This may be because a single growth factor in basal media may not be sufficient to promote high HFF-1 proliferation, thus HFF-1 may require multiple growth factors at the specific concentrations that are present in serum to promote cell growth.
- **2D HUVEC Proliferation: 1.** Maximum HUVEC proliferation was observed in media with serum, followed by bFGF 1 ng/ml. Higher concentrations of bFGF may not promote high cell growth because bFGF may be causing a negative feedback mechanism that discourages proliferation. **2.** There was also significant proliferation on Day 7 with VEGF 10 ng/ml. This may show that VEGF causes a more delayed response.
- **3D HFF-1 Cell Proliferation:** Fibrinogen 10 mg/ml allowed for highest HFF-1 growth. It may be that a lower concentration of fibrinogen allows for more cells to migrate through the porous network and get nutrients from media.
- **3D HUVECs Proliferation: 1.** Fibrinogen 10 mg/ml allowed for highest HUVEC growth. A lower concentration of fibrinogen allows for more cells to migrate through the porous network and get nutrients from media. **2.** It was also noted that HUVECs proliferated less than HFF-1. HUVECs may be more sensitive to a 3D environment and rely more on external signals role for cell growth, while HFF-1 cells are more resilient to their environment.
- **Boyden Chamber:** As the concentration of bFGF increased, the number of HFF-1 cells that migrated to the bottom chamber also increased. This correlation may be due to the increase in growth factor concentration thus allowing for greater chemotaxis from the top to bottom chamber.
- **ICC: 1.** (Data not shown) By Day 7, fibronectin had increased, but no results were observed indicating secretion of collagen. **2.** Results for collagen secretion were similar amongst other groups using the same reagents.
- **FACS: 1.** For HFF-1 grown in 2D, the curves shifted to the left between Day 0 and Day 7 for both α_5 and β_1, indicating decreased integrin expression. That this experiment was run without matrix molecules suggests that the matrix has an effect on integrin expression. **2.** Slight increases for both α_5 and β_1 integrin expression were observed on Day 7 for HFF-1 grown in 3D when compared to Day 0 indicating increased integrin expression. This corroborates the results from the 2D experiment in showing that the presence of a matrix can influence integrin expression. **3.** For HUVECs grown in 3D, there were decreases observed for both α_5 and β_1 integrin expression. It is possible that integrin expression in HUVECs decreases as the cells proliferate.
- **Cytodex Beads: 1.** Sprouting from cells was observed in conditions containing co-cultures with beads. **2.** HUVEC cells on HFF-1 beads may have grown more prominent extensions because HFF-1 cells adhere better to the Cytodex beads and provide HUVECs an excellent environment to proliferate in. Results may have been affected due to not replenishing media in between Day 7 and Day 14.
- **Future Work: 1.** How do growth factors affect integrin expression? **2.** How do HFF-1 cells affect the migration of HUVECs?