Undergraduate Thesis Technical Report

Assessing Microvascular Cell Behavior in an in vitro PEG-DA Hydrogel Cell Culture Assay of Idiopathic Pulmonary Fibrosis (IPF)

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by

Anna Kittel

with

Tara Tavakol

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On my honor as a University student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments.

Anna Kittel

Technical Advisor: Shayn Peirce-Cottler, Department of Biomedical Engineering

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Shayn Peirce-Cottler, PhD, Department of Biomedical Engineering

Assessing Microvascular Cell Behavior in an in vitro PEG-DA Hydrogel Cell Culture Assay of Idiopathic Pulmonary Fibrosis (IPF)

Tara N. Tavakol[1], Anna R. Kittel[1], Samuel Argo[2], Julie Leonard-Duke[1], Lakeshia J. Taite[2], Shayn Peirce-Cottler[1]

[1]Department of Biomedical Engineering, University of Virginia
[2]Department of Chemical Engineering, University of Virginia

Abstract

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive form of pulmonary fibrosis with an average prevalence of about 3.5 cases per 100,000 people and an average survival of 3-5 years after diagnosis; there is currently no cure. IPF entails scar tissue in the lung due to an excessive wound healing response involving extracellular matrix deposition by activated fibroblasts which can threaten integrity of the lung microcirculation by impacting endothelial cell (EC) behavior. ECs respond to both mechanical and biochemical cues, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). It is challenging to deduce how ECs respond to these growth factors, whose levels are altered in IPF, in stiff lung tissue. We used an established hydrogel EC-pericyte co-culture assay with tunable stiffnesses of 2 kPa, 10 kPa, and 20 kPa to study how mechanical stiffness in concert with VEGF or PDGF+FGF affect EC viability and morphology. We hypothesized that stiffness above 10 kPa was sufficient to disrupt EC viability and vessel formation on gels treated with both VEGF and PDGF+FGF. It was seen that vessel formation occurred at all tested stiffnesses, but better network formation was seen at 2 kPa; PDGF+FGF supported better formation than VEGF at all stiffnesses. Greater cell count and cell elongation of ECs was seen at higher stiffness, possibly meaning prolonged wound healing response and abnormal cell characteristics. Projects like this one may provide insight to IPF pathology and contribute to the development of a potential cure.

Keywords: Idiopathic pulmonary fibrosis, hydrogel, endothelial cell, pericyte, stiffness, microvasculature

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic lung condition characterized by proliferation of fibroblasts resulting in excess accumulation of extracellular matrix (ECM) components.[1] This accumulation results in thickening of lung tissue that makes breathing and gas exchange difficult, resulting in lower blood oxygen content and patient discomfort. The causes of IPF are unknown, with this fibrosis occurring in the absence of any obvious illness or injury to the lung. This condition affects around 100,000 Americans, with around 40,000 new cases diagnosed each year, costing patients an average of $26,000 per year.[2,3] Prevalence occurs primarily in middle aged adults, and the median survival is 3-5 years following diagnosis.[4] The cause of IPF is not widely understood, though researchers speculate that genetic and environmental factors both contribute.[5] At a cellular level, endothelial cells, fibroblasts, and pericytes that reside in and nearby the lung capillaries communicate to maintain homeostasis.[6,7] In IPF, this homeostasis is somehow disrupted and the formation of scar tissue in the lung occurs due to an excessive wound healing response involving extracellular matrix

deposition by activated fibroblasts.[8] Endothelial cells likely play a key role in IPF progression due to their proximity to lung capillaries, but this role has not been isolated. In some IPF cases, endothelial cell injury or excess is observed, and this imbalance is related to the excessive wound healing response.[9] It is also believed that fibroblasts contribute to IPF progression through excessive ECM component secretion, which contributes to lung scarring. Another theory is that pericytes, which reside in the lung capillaries, undergo differentiation to become stromal fibroblasts that contribute to ECM deposition.[10] This differentiation typically occurs with injury, but IPF onset occurs in the absence of any obvious injury to the lungs.[11] Partially due to this lack of causal understanding, there is no cure for IPF and current treatments focus on mitigating symptoms and making patients more comfortable for the remainder of their lives. The current standard of care for IPF includes lung transplants, palliative care like oxygen therapy to make breathing easier, and medications for anxiety and pain relief.[12] None of these treatment options work to slow or reverse disease progression, nor are they able to prolong the patient's life. To this end, we aim to **model the progression of IPF lung tissue conditions using polymer hydrogels** that increase in stiffness and growth factor presence to model the progression of IPF. We will then culture human umbilical vein endothelial cells (HUVECs) or co-culture HUVECs and human pericytes from placenta (hPC-PLs) in the varying hydrogel conditions to **determine which factors lead to the greatest morphological changes in cultured cells**. We will primarily look at stiffness and growth factor presence. It is believed that endothelial cells, fibroblasts, and pericytes integrate both mechanical and biochemical cues, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), in ways that contribute to disease progression.[6] VEGF plays a role in development of new blood vessels in the lung microvasculature, FGF helps to regulate cell proliferation and differentiation, and PDGF plays a role in wound healing and blood vessel repair.[13,14] It is possible that these growth factors may contribute to different cell behaviors when observed in conjunction with varying mechanical cues, like surrounding tissue stiffness. These specific aims explore possible contributing microenvironmental factors and their effects on cell morphology and behavior that contributes to IPF progression. Studies like this one may provide insight to the pathology of IPF, which is currently not well understood. Greater understanding of IPF pathology may guide the development of future cures that are able to specifically target IPF at its source to prevent further progression of the disease.

Impact and Innovation

Current IPF research focuses on understanding contributing factors because these must be discovered before cure development. Modeling IPF conditions outside the body using biomaterials or tissue scaffolds is a common way to research what happens at a cellular level.[15] Biomaterials like hydrogels have been studied because they can be easily tuned to mimic physical properties of native lung tissue, like stiffness. This research has failed to recapitulate IPF or positively identify causes, but one theory is that microenvironmental cues cause endothelial cells and pericytes to produce the excess ECM components seen in IPF. 3D in vitro modeling of hydrogels have been established to replicate distinct cellular and molecular signaling pathways that are activated during fibrosis progression. This provides insight into the pathogenesis of the disease and may eventually reveal treatment strategies for IPF lungs.[16] Hydrogels with tunable matrix stiffnesses allow for fibroblast migration, myofibroblast differentiation, and ECM deposition to model deviations in phenotype that exist in IPF and assist with cell-specific therapeutic strategies.[16] One previous study generated ECM hydrogels from control and diseased decellularized human lung tissues that were able to replicate the mechanical properties of native human lung tissue.[17] One shortcoming of this approach is the lack of tunability because the hydrogel is derived from native tissue with established physical properties. Instead of tuning the properties, this study used native healthy and diseased lung tissue samples for comparison. This makes it difficult to control physical and chemical properties of the hydrogel system, which are needed to model and better understand microenvironmental factors that contribute to IPF progression.[18] Our specific hydrogel assay is novel to the field and will allow us to study the impact of two factors (stiffness and growth factor presence) simultaneously on the growth and morphology of endothelial cells and pericytes.

Results

Endothelial Cell Quantification

Preliminary results were obtained from an encapsulation with endothelial cells only (no pericytes) to assess the overall viability of cells within the established hydrogel assay. At time point ten days, it was observed that endothelial cells survived in all stiffness (2, 10, and 20 kPa) and growth factor (VEGF and PDGF/FGF) conditions, supporting an initial hypothesis that this hydrogel-cell culture assay is capable of maintaining cell survival and growth. Brightfield images were taken at day 10 culture time, demonstrating two distinct EC morphologies. ECs were elongated, having a stretched morphology, and circular, having a round morphology (Figure 1).

Using brightfield images, cell count and elongation was

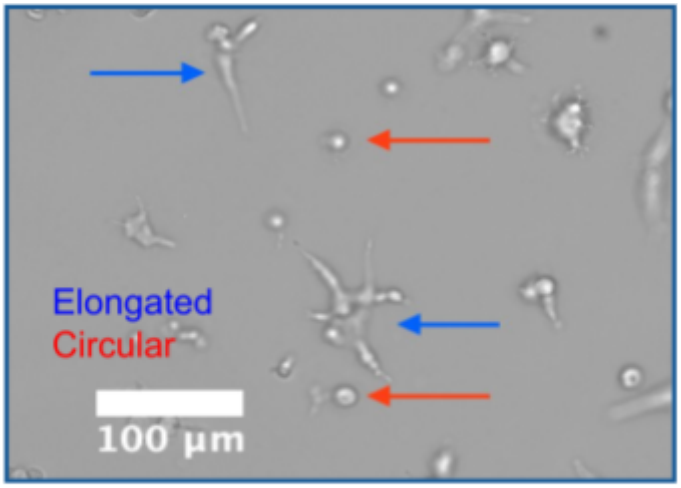


Figure 1. Brightfield image of endothelial cells treated with VEGF at stiffness 2 kPa at 10 day culture time. Circular cells have round morphology and are emphasized with red arrows. Elongated cells have branching morphology and are emphasized with blue arrows.

then measured using ImageJ for both VEGF and PDGF/FGF treated encapsulations (Figure 2). General trends show that both live cell count and number of elongated ECs increases with the increasing hydrogel stiffness for both VEGF and PDGF/FGF conditions. Cell counts at 20 kPa were statistically significantly different than 2 kPa for live and elongated count at both growth factor conditions.

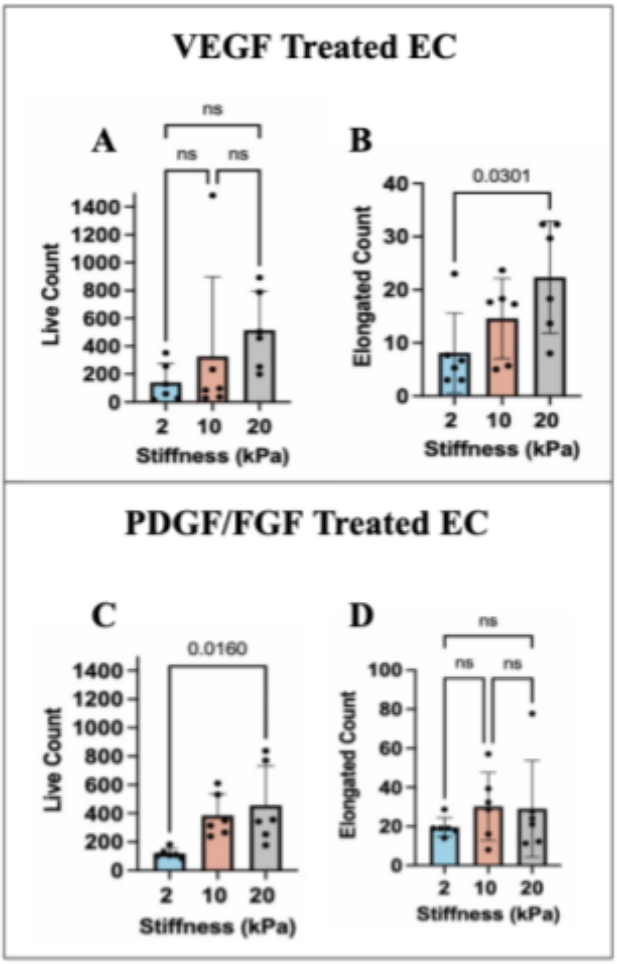


Figure 2. ECs were treated with VEGF or PDGF/FGF at stiffnesses 2, 10, or 20 kPa. For VEGF treated ECs, cell count (A) and cell count of elongated ECs (B). For PDGF/FGF treated ECs, cell count (C) and cell count of elongated ECs (D). Error bars are standard deviation (SD). An ordinary one-way ANOVA with Tukey's post hoc test, n=6, was conducted for each sample.

Immunofluorescent images were obtained from EC-pericyte co-culture samples at culture times 10 and 14 days to qualitatively assess cell behavior, viability, and interactions between ECs and pericytes in the different stiffness and growth factor conditions. At 10 days, it was seen that PDGF/FGF supported better vessel formation than VEGF at all stiffnesses. Within the PDGF/FGF condition it was seen that vessels appeared thicker with increasing stiffness, with 2 kPa having the thinnest vessels and 20 kPa having thicker vessels. VEGF appeared to support clumped cell networks at all stiffness levels (Figure 3).

Immunofluorescence Imaging

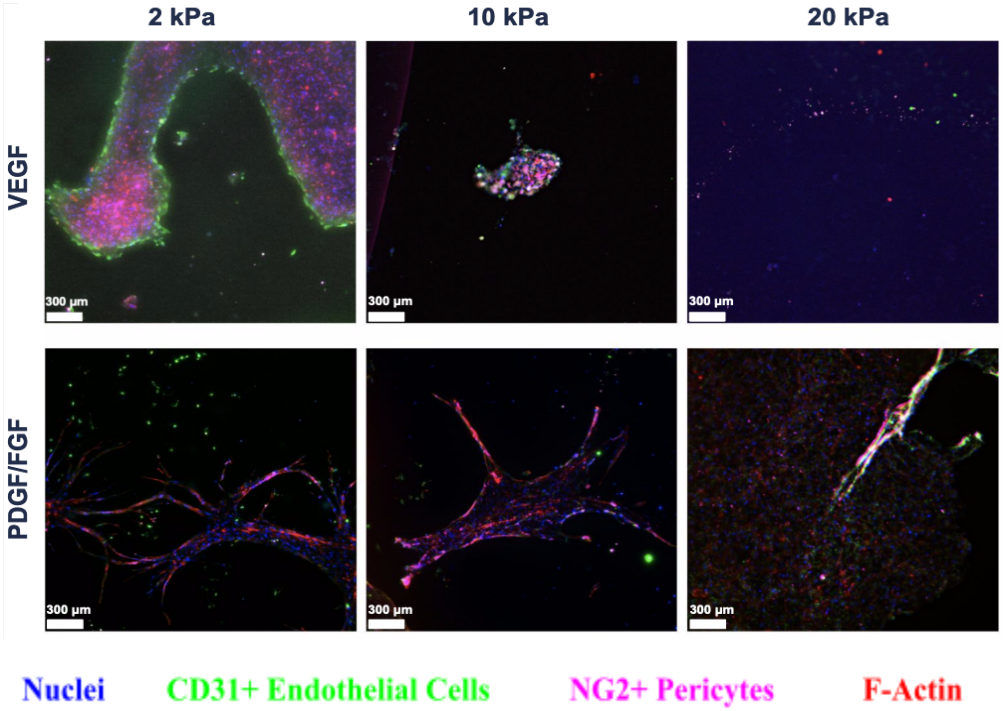
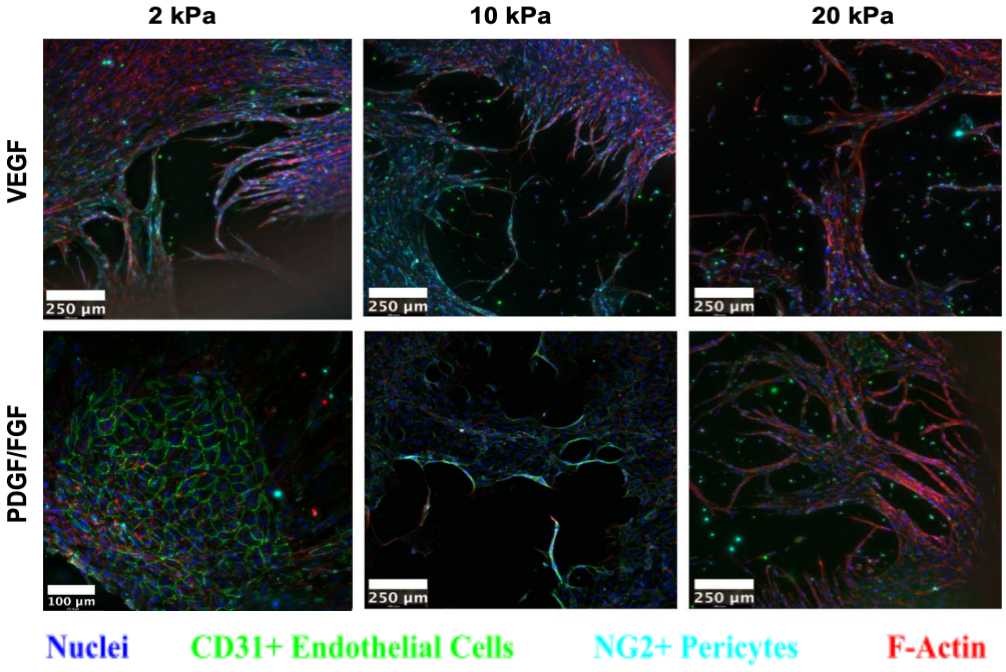


Figure 3. Representative images of overlay of Nuclei (blue), CD31+ Endothelial cells (green), NG2+ Pericytes (magenta), and F-Actin (red) for VEGF or PDGF/FGF at stiffnesses of 2 kPa, 10 kPa, or 20 kPa at culture time 10 days. It was seen that PDGF/FGF supported better vessel formation.

Immunofluorescent images were also obtained at 14 days culture time, where more vessel formation can be seen than at 10 days (Figure 4). Like at 10 days, better vessel formation occurred with PDGF/FGF conditions than VEGF at 14 days. At lower stiffness PDGF/FGF gels, more robust cell networks can be seen, while higher stiffness resulted in thicker vessels. Cell networks in VEGF conditions were more clustered in all stiffnesses, but were more robust at lower stiffness.

Interestingly, vessels were still able to form in 20 kPa gels for both growth factor conditions and greater F-actin (red stain) is expressed at higher stiffnesses. Pericyte-EC interactions can be seen via a relatively even distribution of NG2+ (pericyte) and CD31+ (endothelial cell) expression rather than distinct areas of each individual cell type. Lower stiffnesses PDGF/FGF tend to have more distinct areas of endothelial cells.

Figure 4. Representative images of overlay of Nuclei (blue), CD31+ Endothelial Cells (green), NG2+ Pericytes (cyan), and F-Actin (red) for VEGF and PDGF/FGF at stiffnesses of 2 kPa, 10 kPa, and 20 kPa at culture time 14 days. It was seen that more vessel formation occurred at 14 days than at 10 days.



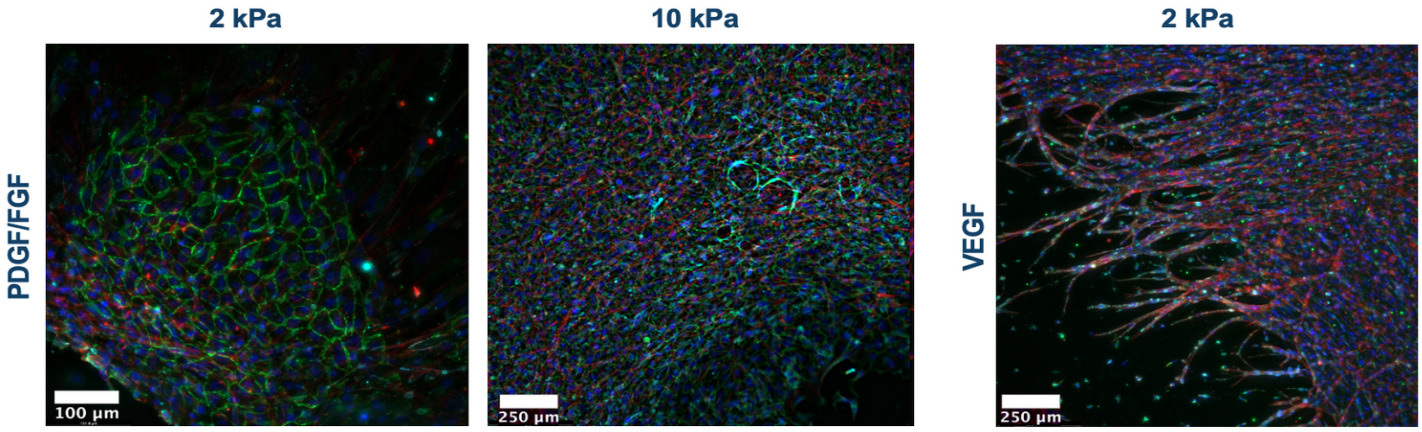


Figure 5. Representative images of overlay of Nuclei (blue), CD31+ Endothelial cells (green), NG2+ Pericytes (cyan), and F-Actin (red) for PDGF/FGF at stiffnesses 2 and 10 kPa and for VEGF at 2 kPa. Cell networks can be seen at lower stiffnesses for both PDGF/FGF and VEGF with more robust vessel network formation seen in PDGF/FGF

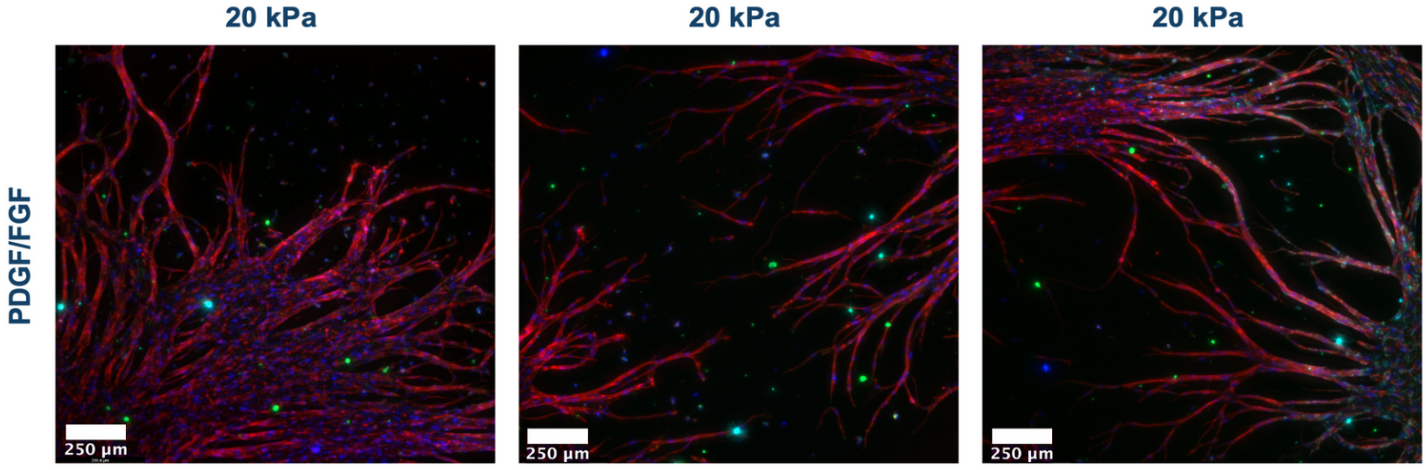


Figure 6. Representative images of overlay of Nuclei (blue), CD31+ Endothelial cells (green), NG2+ Pericytes (cyan), and F-Actin (red) for PDGF/FGF at stiffnesses 20 kPa. Progression of vessel formation can be seen.

Cell networking and vessel formation was further explored in PDGF/FGF samples, looking at networking at lower stiffnesses 2 and 10 kPa and vessel formation at higher stiffness 20 kPa. Endothelial cells can be distinctly seen in green at 2 kPa PDGF/FGF conditions, with nuclei depicted in blue. As expected, more robust cell networks occur at lower stiffness 2 kPa for PDGF/FGF gels. These networks are still present at 10 kPa but it can be seen that they are beginning to be disrupted, forming less cohesive EC networks (Figure 5). At lower stiffness 2 kPa for VEGF conditions, these networks are not as clear; rather, cells form clumped fibrillar networks with greater F-actin expression. Progression of fiber formation was also explored for PDGF/FGF samples at 20 kPa (Figure 6). From left to right of Figure 6, vessels can be seen sprouting from those dense cell networks, reaching out for other vessels, and making connections between cell clusters. These vessels have high levels of F-actin expression, seen in red fluorescence. Distinguishment between endothelial cells (green) and pericytes (cyan) is less clear due to this high expression of F-actin, but cellular nuclei can still be seen in blue.

Discussion

This project successfully developed a cytocompatible *in vitro* hydrogel cell-culture assay capable of promoting endothelial cell and pericyte growth and survival for 14 days. The incorporation of both different growth factors PDGF/FGF or VEGF in conjunction with different stiffness levels (2, 10, and 20 kPa) allowed for a more

controlled environment to observe how these conditions impact the behavior and interactions of endothelial cells and pericytes. The inclusion of two time points, 10 and 14 days, allowed for the observation of progression of vessel formations and how interactions between ECs and pericytes might change over time. More vessels were seen at day 14 than at day 10, illustrating that the cultured cells continued to proliferate and differentiate between 10 and 14 days. This finding supports our initial hypothesis that this hydrogel-cell culture assay would be cytocompatible.

Endothelial Cell Count and Elongation

Quantification of cell count of live and elongated endothelial cells revealed that higher stiffness 20 kPa conditions resulted in greater number of live and elongated endothelial cells for both PDGF/FGF and VEGF conditions. Greater number of live cells in higher stiffness gels could mean uncontrolled endothelial cell proliferation as a result of the prolonged wound healing response seen in IPF. Greater number of elongated endothelial cells in higher stiffness gels reflects abnormal cell morphology, which is likely occurring with abnormal behaviors.

Cell Networks and Vessel Formation

Another key finding of this particular assay is that the growth factor PDGF in conjunction with FGF supported far better cell networks and vessel formation than the VEGF conditions at all stiffnesses. This an interesting finding because VEGF is involved in promoting the development of new blood vessels while PDGF plays a role in wound healing and blood vessel repair and FGF helps promote cell proliferation and differentiation.[13,19,20] The use of FGF with PDGF may have created a synergistic effect resulting in greater blood vessel formation than VEGF alone. At lower stiffness 2 kPa PDGF/FGF conditions, robust cell networks were seen; endothelial cells formed dense and cohesive networks with minimal gaps. These networks could still be seen at 10 kPa but were less robust, potentially forming leaky networks. At 2 kPa VEGF conditions, cell networks could also be seen but with thick, clumped vessels forming off the edges of these networks. At higher stiffnesses 10 and 20 kPa VEGF conditions, less cell networks can be seen and instead many clumped vessels are noticeable. At higher stiffness PDGF/FGF and VEGF conditions, greater F-actin expression can be seen and there is less distinct EC and/or pericyte fluorescent signal due to this large volume of F-actin signal. Greater F-actin expression is characteristic of new vessel formation because F-actin is a component of cell cytoskeletons that plays a role in cell motility and contraction. During vessel formation, endothelial cells take on stretched morphologies, with pericytes wrapping around them; it is expected that greater F-actin expression would be seen with this morphological change. It was interesting that these vessels could be seen at higher stiffness 20 kPa conditions, but it could be a compensatory response to the increased environmental stiffness. The vessels seen in higher stiffness conditions are thick and clumped, not representative of the morphology of vessels you would expect in healthy lung tissue.

Conclusions

The outcomes of this research have provided insight on the cellular behavior of lung microvasculature in response to conditions that are similar to IPF. Though this project was not able to definitely determine the pathology of IPF, interesting outcomes were observed for cellular behavior in the different stiffness and growth factor conclusions. Based on the findings of this research, we further hypothesize that the increase in stiffness seen during IPF progression results in abnormal interactions between endothelial cells and pericytes in lung microvasculature. Lower stiffnesses characteristic of healthy lung tissue supported the formation of robust cell networks, while greater stiffnesses appear to result in overactive endothelial cells and pericytes that form thick, clumpy, and potentially leaky microvasculature structures. More cell networks and vessels could be seen at 14 days than at 10 days, meaning cells continue to proliferate and differentiate after 10 days. These trends could continue even beyond the time point of 14 days; resulting in even more cell networks or vessels beyond 14 days. IPF likely results in abnormal cell characteristics, uncontrolled cell proliferation, and abnormal interactions between different cell types involved in the microvasculature.

Future Directions

This study could be further replicated with the incorporation of time-course imaging around culture time 10 to 14 days. Vessels were seen at 14 days culture time that were not seen at 10 days, so it would be

interesting to hone in on this time period and to observe the outgrowth of these vessels over time. Further, stains N-cadherin and VE-cadherin could be incorporated to observe endothelial cell-endothelial cell and endothelial cell-pericyte junctions respectively. This would provide greater insight to the cell interactions occurring with the cell networks and vessel formations and how these are disrupted with the increasing stiffness. Vessel dimensions could also be further analyzed to find vessel diameters, lengths, and distance between vessels.

Materials and Methods

Hydrogel Encapsulation

PEG-DA hydrogels were prepared with stiffnesses of 20 kPa, 10 kPa, and 2 kPa using Lys(alloc)-OH as a competitive monomer, which allows for altering stiffness of the hydrogel.[21] This range of stiffness was chosen to model IPF progression, as 2 kPa reflects healthy lung tissue, 10 kPa reflects IPF onset, and 20 kPa reflects IPF conditions. Hydrogels contained 0.7 nmol/mL soluble and PEG-conjugated growth factors, either VEGF or the combination of PDGF/FGF. Human umbilical vein endothelial cells (HUVECs, Lonza) were cultured at 20,000 cells per well and human pericytes (hPC-PL, PromoCell) were cultured at 2,000 cells per well with EBM-2 media (Lonza), which was changed every other day. These cell densities were chosen from previous encapsulation protocols in the lab. Samples were maintained in 24-well plates for 10 or 14 day culture times in a CO2 incubator kept at physiological conditions and contained cells seeded on top of the hydrogel.

Immunofluorescent Staining

At day 10 and 14 culture time, fixing and staining protocol was done to prepare the samples for imaging. The cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed with phosphate buffered saline (PBS) 3x at 5 minutes each, then permeabilized for 30 minutes with 0.5% Triton X-100. Following 3 more 5 minute PBS washes, samples were blocked with 5% donkey serum in 3% BSA overnight at 4C. Following the overnight incubation, the gels were washed 3x with PBS for 1 hour each then incubated with primary antibodies CD31 to stain HUVEC cytoplasm and NG2 to stain pericyte cytoplasm for 48 hours at 4C. Following incubation, gels were rinsed in 0.5% donkey serum for 1 hour, followed by 2x PBS washes for 30 minutes each. Samples were then stained with matching secondary antibodies Donkey-anti-mouse-IgG 488 and Donkey-anti-rabbit-IgG 647 for 24 hours at 4C. The gels were then rinsed with 0.01% Tween and incubated with phalloidin 546, which detects F-actin, and NucBlue, or DAPI, which detects DNA in nuclei. Immunofluorescence imaging was done to amplify antibody signals using a Leica Thunder microscope.

Image Quantification

Image quantification was performed using ImageJ software. Endothelial cells of stiffnesses 2 kPa, 10 kPa, and 20 kPa for VEGF and PDGF/FGF conditions were counted using the cell counter plugin on ImageJ. Number of elongated cells were counted using the cell counter plugin, with counting focused on cells with stretched out morphology. Number of circular cells were also counted using the cell counter plugin, where cells that appeared as circles were counted.

End Matter

Author Contributions

Anna Kittel and Tara Tavakol prepared and maintained cell culture of endothelial cell and pericyte hydrogel co-cultures. Anna Kittel and Tara Tavakol performed gel immunostaining and immunofluorescence microscopy. Tara Tavakol performed ImageJ analysis to quantify life and elongated cell counts. Anna Kittel and Tara Tavakol contributed equally to the writing of this final manuscript.

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