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14. ABSTRACT

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a hereditary disorder associated with a mutation of either PKD1 or PKD2. The pathophysiological mechanisms of ADPKD which ultimately result in the formation of bilateral focal cysts are not well understood. Specifically, the early disease pathways associated with the transition for a tubule to cystic phenotype are not well understood and consequently no targeted treatments for ADPKD exist. The purpose of this project is to develop and characterize a physiologically relevant platform that combines a 3D culture environment, which allows for changes in morphological phenotype, and fluid induced shear stresses that mimic the mechanical forces found in the kidney. The focus of the research was specifically targeted around developing the perfusion system and characterizing the cell types necessary for developing an in vitro perfusion model of ADPKD. The ultimate goal of the research is to characterize normal and disease phenotypes within the model system.

15. SUBJECT TERMS

ADPKD, perfusion bioreactor, three dimensional, tissue engineering

Table of Contents

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	10
5. Changes/Problems	11
6. Products	11
7. Participant & Other Collaborating Organizations	11
8. Special Reporting Requirements	11
9. Appendices	12

1. Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a hereditary disorder associated with a mutation of either *PKD1* or *PKD2*. The pathophysiological mechanisms of ADPKD which ultimately result in the formation of bilateral focal cysts are not well understood. Specifically, the early disease pathways associated with the transition for a tubule to cystic phenotype are not well understood and consequently no targeted treatments for ADPKD exist. The purpose of this project is to develop and characterize a physiologically relevant platform that combines a 3D culture environment, which allows for changes in morphological phenotype, and fluid induced shear stresses that mimic the mechanical forces found in the kidney. The focus of the research was specifically targeted around developing the perfusion system and characterizing the cell types necessary for developing an *in vitro* perfusion model of ADPKD. The ultimate goal of the research is to characterize normal and disease phenotypes within the model system.

2. Keywords

ADPKD, perfusion bioreactor, three dimensional, tissue engineering

3. Accomplishments

What were the major goals of the project?

	Major Goals	Timeline	Percent Completion
1	Design of the perfusion system, prototyping and fabrication	1-6	100%
2	Silk scaffolding incorporation into the perfusion system	3-5	100%
3	Immortalized human renal cortical epithelial cell seeding optimization in the perfusion system with respect to flow rate, cell density and seeding duration	6-10	100%
4	Insertion of tet-pLKO-pkd1 shRNA into immortalized normal human cortical kidney cells	1-5	95%
5	Incorporation of PKD1 knockdown cells into a 3D collagen/matrigel environment	6-10	100%
6	Cyst characterization in microperfused normal and diseased (PKD1 deficient) tissues at normal and injury based flow condition	18-24	40%
7	Phenotypical and functional assessment of microperfused normal and diseased tissues at normal and injury based flow conditions	24-30	30%

What was accomplished under these goals?

- 1) Design of the perfusion system, prototyping and fabrication:** As previously reported, in order to function as a model of ADPKD, the perfusion system needed to have both a 3D bulk component and physiologically relevant shear stresses across the epithelial cell surface. A primary component of the proposed perfusion system design was the inclusion of a porous silk protein scaffold capable of having extracellular matrix, specifically a 50:50 mix of 1 mg/ml type 1 collagen and matrigel, infused within the

bulk. Initial bioreactor designs were focused on scaffold fabrication within rectangular wells cut out of PDMS (polydimethylsiloxane) with 250 micron wires fed across the well in order form channels within the scaffold bulk. Despite the ability to form channeled, salt-leached silk scaffolds within the wells, the porosity and channel integrity were found to be inconsistent, with low reproducibility. This design was particularly limiting considering a compromised channel structure would significantly affect the ability to accurately model the shear stresses at the epithelial cell surface. As such, we designed a novel system where a porous silk scaffold could be pre-seeded with a confluent layer of renal epithelial cells and, loaded into the top of the bioreactor and subjected to uniform shear stresses.

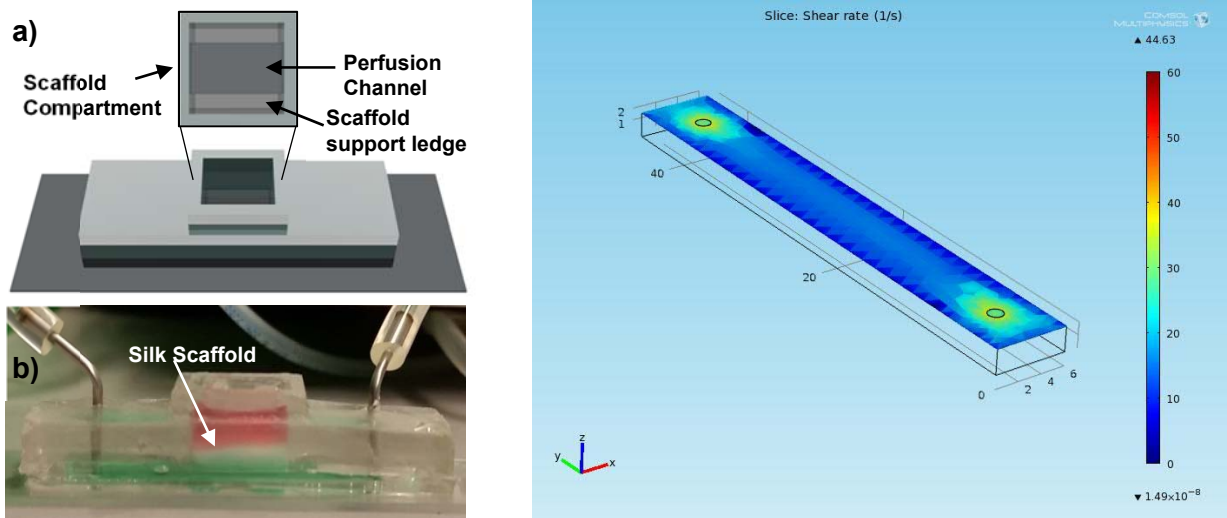


Figure 1. (a) AutoCAD model of the 3D perfusion bioreactor. The silk scaffold dimensions are several millimeters wider than the channel dimensions to allow for stable scaffold incorporation. (b) Photograph of the final assembled PDMS bioreactor. (c) COMSOL model of shear rate at the height of the scaffold within the channel.

The top loaded bioreactor was designed using AutoCAD and the system was optimized based on concurrent COMSOL modeling. The final design depicted in Figure 1 allows for the bottom of a 12 x 10 mm silk scaffold to be exposed to flow through the 44 x 6 x 2 mm channel. The bioreactor channel, port location and scaffold placement were optimized for undisrupted, laminar flow at the scaffold location. In addition to the original design parameters the final bioreactor is capable of supporting different media conditions on each side of the scaffold as depicted in Figure 1b with the green and red dyes. This capability further enhances the utility of the bioreactor to sustain complex tissue models where additional cell types and basolateral access is desirable. COMSOL modeling, as shown in Figure 1c, was used to verify uniform shear stress at the surface of the scaffold. The completed bioreactor is capable of exposing the epithelial cells to the proposed range of shear stresses that are necessary for mimicking normal and injury based flow conditions.

Repeatable device fabrication was accomplished using a custom multistep fabrication process. A negative mold of the bioreactor, designed in AutoCAD, was created using a Stratasys 3D printer. PDMS was cast in the negative molds in order to create permanent biocompatible plastic masters (SmoothCast 310).

All goals of task one were met resulting in the fabrication of a well characterized 3D perfusion bioreactor capable of reaching physiologically relevant shear stresses. Due to the dimensions of the channel high flow rates will be necessary to reach the desired shear stresses. To minimize media consumption, we have

been cycling media through the system using a peristaltic pump. The resulting bioreactor is not only well suited for the proposed ADPKD tissue model but complex tissue systems requiring multiple cell types and media conditions.

- 2) *Silk scaffolding incorporation into the perfusion system:* The final bioreactor design significantly reduced the complexity of the scaffold design by eliminating the need to form a channel through the porous bulk. The channeled scaffolds were observed to have less structural integrity and the use of the wire to form the channel had a significant impact on the adjacent pores. For

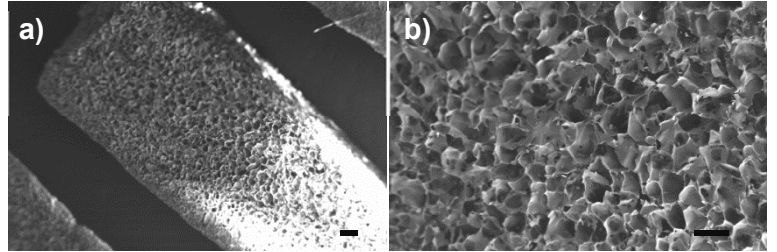


Figure 2. Scanning electron microscopy images of the cross section of a lyophilized silk scaffold. Scale bar (a) 200 microns (b) 100 microns.

the current 3D perfusion system the chosen method for silk scaffold fabrication consists of freezing 5% aqueous silk at -20°C and lyophilizing. By freezing the silk solution in a 24 well plate we are able to fabricate scaffolds with a flat bottom and minimal surface defects on the side which will be exposed to flow. All scaffolds are cut to a size of 12 x 10 x 2 mm. Scaffold pores within bulk are approximately 90 microns in diameter (Figure 2). The scaffolds are sterilized via autoclave and dried using a vacuum before infusing with Matrigel/collagen. Based on the final bioreactor design the silk scaffolds can be easily placed into the perfusion system inside the reservoir compartment and on top of the scaffold supports.

- 3) *Immortalized human renal cortical epithelial cell seeding optimization in the perfusion system with respect to flow rate, cell density and seeding duration:* Similar to the scaffold optimization, the top loading bioreactor design significantly increased the reproducibility and ease of cell seeding. As opposed to most tradition perfusion systems the cells can be pre-seeded on the scaffold and subsequently placed under flow conditions. This approach significantly increases the usability of the system by eliminating the need to determine flow based seeding parameters. To establish a confluent cortical epithelial cell layer on the scaffold surface, 5×10^5 cells are added on top of each ECM infused scaffold within 12 well plates. An even layer of renal cortical epithelial cells has repeatably been established on the surface of the silk scaffold and has been shown to be viable for at least 8 weeks in static culture (Figure 3). Cell seeding can be characterized on the scaffold surface using an inverted microscope without the need for paraffin or cryo embedding.

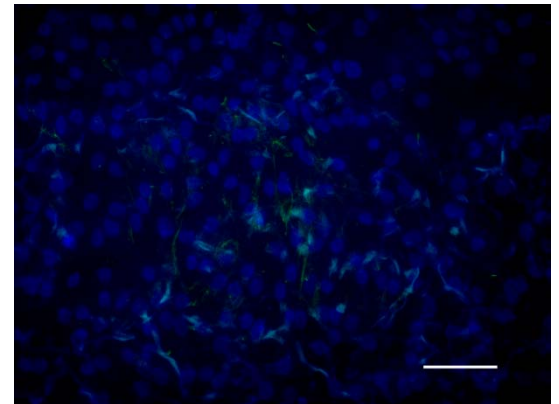


Figure 3. Renal cortical epithelial cells (RPTEC/tert1) on the surface of the porous silk scaffold at week 8. (Blue - Dapi, Green - Acetylated alpha tubulin, Scale - 50 microns)

4) *Insertion of tet-pLKO-pkd1 shRNA into immortalized normal human cortical kidney cells:* To establish an inducible knockdown of *PKD1*, *PKD1* shRNA was inserted into a *pLKO-tet-on* plasmid. Plasmids were transfected into immortalized renal cortical epithelial cells (NKi-2). A range of doxycycline concentrations were dosed on the cells for 48 hours to test for induction of the knockdown. qPCR analysis revealed a reduction in *PKD1* in all concentrations between 50 and 200 ng/ml (Figure 4). While a reduction in PKD1 is observed we were unable to achieve a complete knockdown of PKD1 expression.

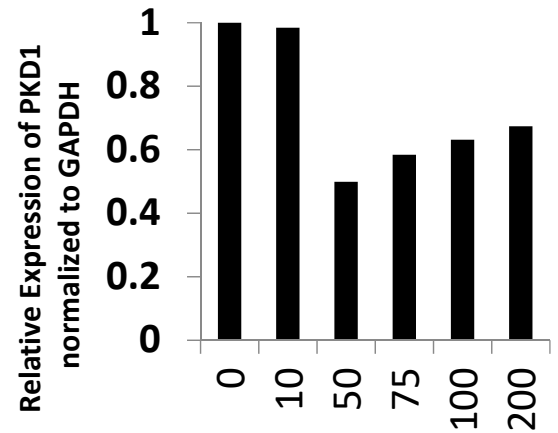


Figure 4. qPCR for PKD1 expression 48 hours after doxycycline treatment.

After we were unable to achieve a complete knockdown of PKD1 expression using the *tet-pLKO-pkd1 shRNA* we sought additional experimental approaches. These included utilizing an alternative immortalized renal cortical epithelial cell line (RPTEC/tert1 from ATCC) and advances in CRISPR/Cas9 to knockout PKD1. This cell line was chosen due to previous characterization with respect to cilia expression and epithelial polarization. In preliminary experiments co-transfection of the knockout plasmid (figure 5) with the polycystin-1 homology directed repair plasmid was achieved at low efficiencies. This inefficiency of transfection has limited our ability to achieve a stable population of RPTEC/tert1 cells with the knockout due to low viability of this cell type at low concentrations. Attempts to optimize this knockout include using electroporation and conditioned media at low cell concentrations.

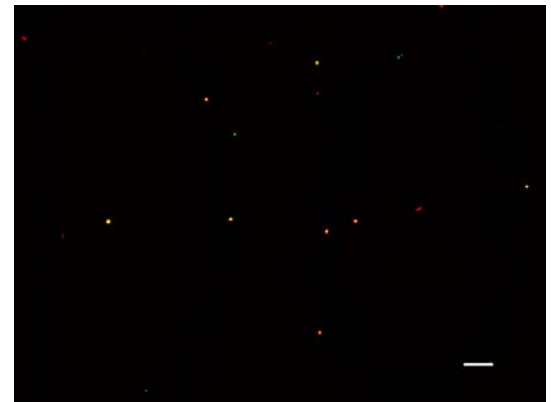


Figure 5. RPTEC/TERT1 cells 48 hours after transfection with a polycystin-1 CRISPR/Cas9 knockout plasmid (GFP positive cells) and a polycystin-1 homology directed repair plasmid (RFP positive cells). Yellow cells indicate co-transfection (scale bar is 200 microns).

5) *Incorporation of PKD1 knockdown cells into a 3D collagen/matrigel environment:* Based on previously established protocols in our lab, we established the static 3D cultures of renal epithelial cells for 2 weeks before induction of the knockdown. In initial studies, after 3 weeks of doxycycline treatment at concentrations of 50 and 100 ng/ml we observed a dilation of the tubular structures at the edges of the gel in the 100 ng/ml after staining with H&E (data not shown). Treatment of cultures with the cAMP agonist Forskolin did not produce notable changes in the histology of the knockdown tissues. However, tubule dilations were observed in some of the normal cultures treated with Forskolin. Due to the localization of

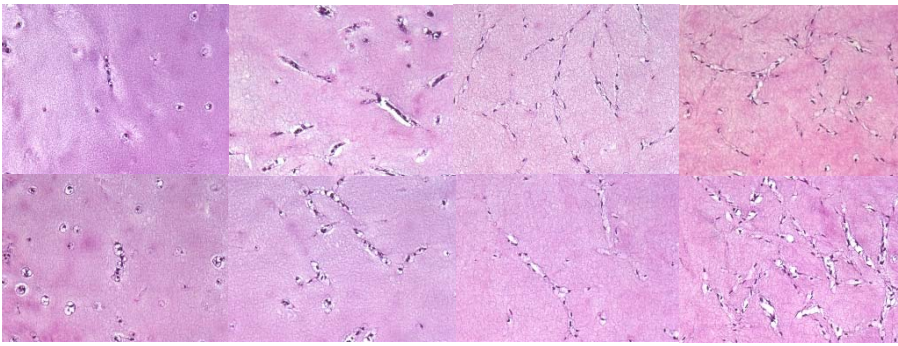


Figure 6. Renal cortical epithelial cells after 5 weeks of culture (3 weeks of treatment) at 1.0 mg/ml (a & e), 1.2 mg/ml (b & f), 1.5 mg/ml (c & g) and 1.8 mg/ml (d & h). e-h) Samples were treated with 100 ng/ml of doxycycline for 3 weeks (Scale bar – 100 microns)

the phenotypic outcome to a specific region of the tissue culture it was hypothesized this difference was the result of either a higher collagen concentration or a higher stiffness at the edge of the gel. Culture optimization experiments assessing an array of collagen concentrations (1 mg/ml, 1.2 mg/ml, 1.5 mg/ml and 1.8 mg/ml) mixed 50:50 with Matrigel/collagen resulted

in a greater amount of tubule dilation in cultures with higher collagen concentrations (Figure 6). While these treatments and matrix optimization yielded the dilated phenotype cyst formation was not achieved in static culture.

In expanding the toolkit to include an additional immortalized renal epithelial cell line we also conducted experiments to characterize the morphology of these cells in 3D culture. RPTEC/tert1 cells did not form structures under the same extracellular matrix conditions as the NKi2 cells (data not shown). When embedded in Matrigel/collagen type1 these cells did not remain viable. However, it was discovered that embedding RPTEC/tert1 cells in Matrigel within a lyophilized silk sponge we were able to achieve a cystic phenotype (figure 7). The formation of cysts within the pores of the silk sponge but not in the ECM only conditions further suggests that ECM mechanics such as stiffness play a role in kidney structure formation *in vitro*.

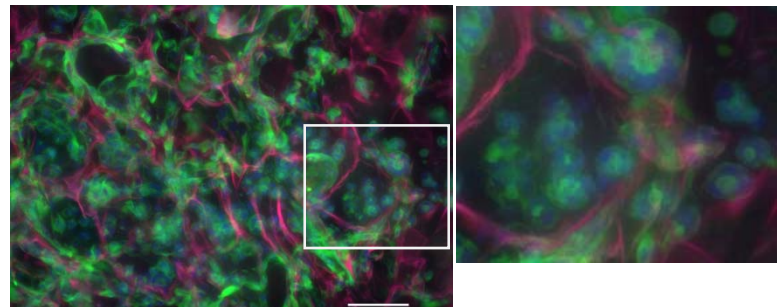


Figure 7. RPTEC/tert1 cells embedded in Matrigel within lyophilized silk sponges. Within the pores the cells form multicellular cysts. AdipoRed is used as a counterstain for the silk scaffold. (Blue- DAPI, Green- phalloidin, Red – adipored Scale bar – 100 microns)

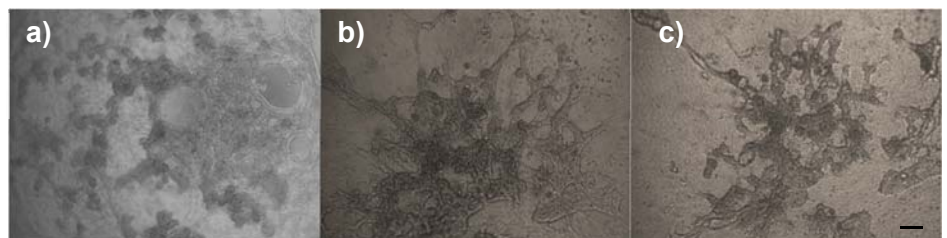


Figure 8. Brightfield images of RPTEC/tert1 cells after 48 hours of hanging drop culture (a) and subsequent embedding in a Matrigel/collagen hydrogel at day 2 (b) and day 12 (c).

Based on clustering of RPTEC/tert1 cells in 2D culture we also hypothesized that increase cell proximity would promote cell viability in 3D culture. To test this hypothesis we established a protocol for clustering the cells via hanging drop culture (80,000 cells in a 10 μ l droplet) followed by embedding in a Matrigel/collagen ECM hydrogel (figure 8). Hanging drop culture of RPTEC/tert1 cells produces a disperse clusters as opposed to a single solid pellet (figure 8a). When these cultures are embedded in a 3D

environment they produce tubule-like extensions that remained viable out to 12 days of culture. Accordingly, we have developed a new toolkit for culturing RPTEC/tert1 cells that yields both cystic and tube-like morphologies that are valuable for future ADPKD disease models.

- 6) Using the previously described perfusion bioreactor and established seeding conditions we compared scaffolds perfused at normal shear stress (0.2 dyn/cm^2) to scaffolds maintained statically. The static scaffolds had a greater number of stress fibers and more diffuse actin staining compared to the scaffolds under normal conditions (figure 9a).

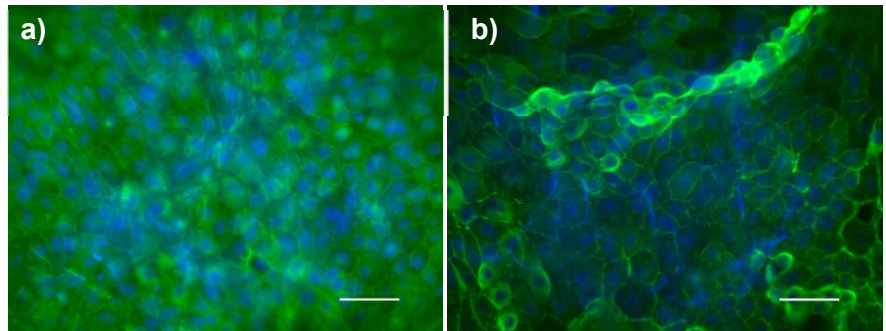


Figure 9. RPTEC/TERT1 cells on lyophilized silk scaffolds infused with Matrigel/collagen after static (a) and perfusion conditions with a shear stress of 0.2 dyn/cm^2 for 72 hours (Blue - Dapi, Green - phalloidin , Scale - 50 microns)

Meanwhile, the scaffolds exposed to normal shear stress demonstrated localization of f-actin to the cell junctions suggestive of greater tight junction formation. These results support the validity of this system as a method for studying the effect of shear stress on kidney epithelial cells seeded on a 3D scaffold. Future experiments will continue to analyze cell behavior at higher shear stresses as well as low shear. Perfusion studies were not completed on disease cells due to on-going efforts to fully characterize the phenotype of the RPTEC/tert1 cells in 3D.

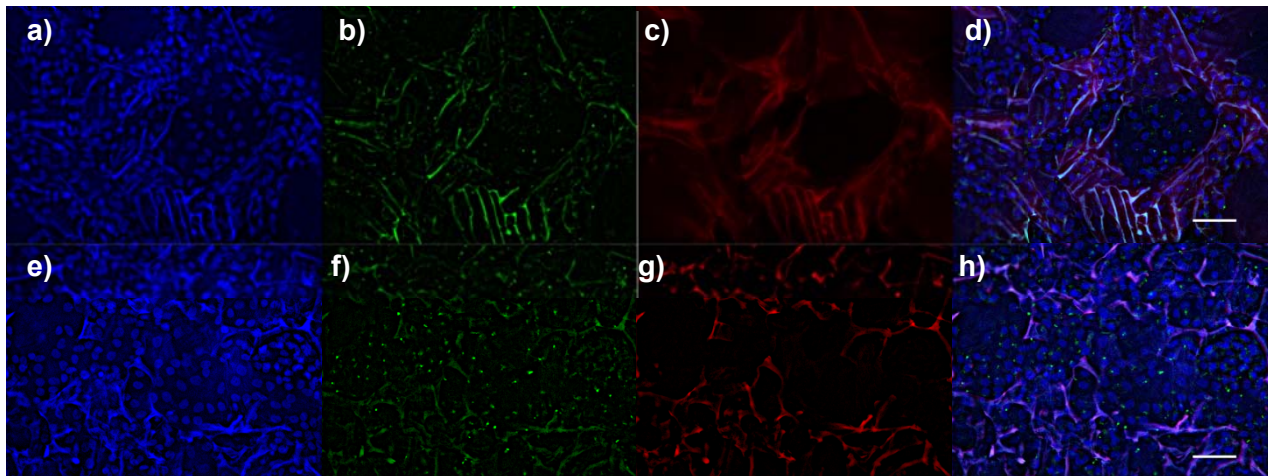


Figure 10. Full focus fluorescence images of RPTEC/TERT1 cells on lyophilized silk scaffolds infused with Matrigel/collagen after static (a-d) and perfusion conditions with a shear stress of 0.2 dyn/cm^2 for 72 hours (e-h) (Blue - Dapi, Green -acetylated alpha tubulin, Scale - 50 microns)

- 7) Additional analysis of the scaffolds under static and perfused conditions was conducted under the same conditions as goal 6). Expression of primary cilia is particularly relevant to studies of ADPKD due to the localization of polycystin-1 on the primary cilia and its role as a mechanosensor. The increased expression of primary cilia has been used to suggest the presence of a more differentiated phenotype of proximal tubule cells in kidney microfluidic devices. However, the length of the cilia has also been shown to shorten

under normal flow and lengthen in response to injury. Staining for acetylated alpha-tubulin expression in RPTEC/tert1 under static and flow conditions verified the maintenance of ciliary expression seen in 2D with a possible increase under fluidic conditions (figure 10). Further experiments will be conducted to examine ciliary length in response to flow conditions in order to determine how both flow alters cilia expression when cells are grown on a 3D scaffold.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

4. Impact

What was the impact on the development of the principle disciplines of the project?

The 3D perfusion platform presented here consists of a simple, modular design that is readily adaptable to a range of tissue models. This approach enables cells to be exposed to fluid forces while growing on a surface that mimics the structural environment in the body. Unlike standard two dimensional cell culture approaches the use of a 3D environment allows for the incorporation of multiple cell types. The inclusion of both the 3D protein environment and fluid flow will enable the development of more physiologically relevant tissue models for studying disease and drug development. Moreover, 3D tissue culture using this perfusion platform can be achieved with minimal technical difficulty due to ability to reliably grow cells on a structurally stable scaffold before being placed within the perfusion system. The developed 3D perfusion platform can be applied to numerous different tissue models requiring the inclusion of fluid based stimulation, such as the kidney and vasculature, and as a result the platform has the capability of having a significant translational within the field of tissue engineering. This system is currently being utilized to study the effect of fluid forces on cystic disease development within the kidney. This approach can offer new insights into possible early therapeutic treatments to limit the progression of the disease.

Moreover, throughout this project we further developed methods for growing kidney cells that more closely mimic the structures within the body. These 3D models will enable us to study how these structures change in response to disease and treatment.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. Changes/Problems

Changes in approach and reasons for change

As described above, the final bioreactor design was different from the originally proposed design due to issues with structural integrity that compromised the laminar flow through the scaffold. Despite the slight shift in design the final bioreactor design was able to achieve uniform shear stresses on the surface of a silk scaffold capable of capturing for morphological in the epithelial cell layer.

Due to the inability to achieve a cystic phenotype from the cells that we originally established the PKD1 knockdown we began working with a commercially available immortalized cell line with similar characteristics to our original cortical epithelial cells. The incorporation of an additional cell line required addition optimization of 3D culture similar to what was outlined in the proposal.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

6. Products

Presentations

(Poster) Kimmerling, E.P, Coburn J., & Kaplan, D.L (2015). A Modular, Three Dimensional Perfusion Bioreactor. TERMIS World Congress. Boston, Massachusetts.

Publications

Kimmerling, E.P, Coburn J., & Kaplan, D.L. A Modular, Three Dimensional Perfusion Bioreactor. (*in preparation*)

7. Participant & Other Collaborating Organizations

None

8. Special Reporting Requirements

None

9. Appendices
none