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In Vitro Modeling of the Kidney for Drug Transport and Toxicity Testing

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In Vitro Modeling of the Kidney for Drug Transport and Toxicity Testing

by

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DISSERTATION

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I live my life in widening circles
that reach out across the world.
I may not complete this last one
but I give myself to it.

I circle around God, around the primordial tower.
I've been circling for thousands of years
and I still don't know: am I a falcon,
a storm, or a great song?

- Rainer Maria Rilke
Book of Hours, I 2

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night with my need to talk. She is quite literally my favorite person in the world. I would also like to thank my grandmother who loves me selflessly, takes pure joy in all my achievements and showed me, by example, how to be a strong, independent woman. This list is not complete without including my best friend Aparna Jayaraman, who is, for all relevant purposes, truly family. Aparna has stuck with me through all the roughest parts of grad school, listening patiently to all my doubts, fears and frustrations and always reminding me of my own worth. I'm grateful to her for never judging me, always making time for me and helping me develop my wild side.

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ABSTRACT

It is important to accurately model drug disposition in the preclinical setting before novel drug compounds are introduced into humans. However, it is evident from current statistics on the drug development process that there is much room for improvement in the preclinical assessment of drug candidates in order to avoid clearance or toxicity issues when the drug is used in the clinic. One organ that plays a significant role in drug disposition and is therefore important to model is the kidney, which filters blood and is responsible for elimination of over one third of all drugs. The focus of this dissertation was to improve in vitro modeling of the kidney. First, multiple proximal tubule cell lines were systematically compared in order to identify the most physiologically relevant line. It was found that while the human cell lines hold some promise, non-human transfected cell lines remain more robust models of proximal tubule drug transport. Compared to the human cell lines, non-human transfected cell lines have superior monolayer formation, higher levels of transporter expression and function and are easier to grow in culture. Second, the role of one particular aspect of the *in vivo* environment, shear stress, was studied to better understand the effect on drug transporters and the possible biological pathways involved. Results indicated that shear stress affects active transport of organic cations and expression of relevant transporters in renal cells in a cilia dependent manner. Lastly, the design of a 1:1000 scaled model of the kidney that can be incorporated into a 1:1000 scaled human-on-a-chip system is presented along with the results of initial prototyping and future directions. In summary, the studies in this dissertation provide insight into the limitations of existing renal cell models, expand our current knowledge of the interaction between the cellular environment and drug transport functionality and provide a foundation for the future development of a scaled kidney on a chip module.

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INTRODUCTION

Drug development is a long and expensive process that has a very low success rate. Despite improved methodology for preclinical assessment of drug candidates, undesirable clearance or toxicity is still sometimes observed when a drug enters the clinic. Approximately 30% of drugs that are successful in preclinical studies fail in humans due to unanticipated clearance or toxicity issues[1]. Additionally, many potentially useful drug candidates are likely withdrawn before clinical trials due to methods that inaccurately predict drug disposition. Much of this can be attributed to limitations of current preclinical models.

One organ that is important for preclinical modeling of drug clearance and toxicity is the kidney, which is responsible for elimination of over one third of all drugs. Approximately 10-20% of drug candidates are withdrawn during clinical trials due to inaccurate renal clearance or toxicity predictions[2,3]. Additionally, over 20% of acute kidney injury (AKI) is caused by nephrotoxicity from approved drugs such as cisplatin and tenofovir[4]. This number increases to 66% in older adults due to other overlapping conditions and drug-drug interactions in patients on multiple medications[4]. While acute kidney injury is often reversible, it can still be expensive, require multiple hospitalizations and in severe cases, result in permanent damage and death. Clearly, there

is a need for better models of the human kidney in which new drugs as well as more patient-specific dosing conditions can be tested.

The kidney is a complex organ that is made up of about one million nephrons, each of which consist of several segments arranged in series that work together to filter blood and produce urine (Fig 1.1A[5]). Blood first enters the glomerulus, where cells and large proteins are excluded and the remaining plasma is filtered through. This filtrate then passes through the proximal tubule (PT) where water and nutrients are reabsorbed and drugs and other toxins are secreted via active transport. Following this, the filtrate goes through the loop of Henle, the distal tubule and the collecting ducts where water and salts are reabsorbed, resulting in concentrated urine that is directed into the bladder. Of these components, the proximal tubule cells are most important for drug studies because they perform the majority of drug clearance and are responsible for >90% of nephrotoxicity from drugs[6]. The tubule is a monolayer of epithelial cells with filtrate flowing across the apical surface. A basement membrane underlies the tubular epithelium and adjacent peritubular capillaries allow for reabsorption. The environment experienced by proximal tubular cells is quite complex with constant exposure to fluid shear stress and apicobasal oncotic gradients.

REQUIREMENTS FOR A KIDNEY MODEL

An ideal model of the proximal tubule would include cells that can replicate the functions of those found *in vivo* and incorporate as many aspects of the *in vivo* environment as possible.

Proximal Tubule Cells:

There are several requirements for proximal tubule cells to be incorporated into a kidney model. The cells need to maintain a cuboidal morphology, express tight junctions along the edge of the cell membrane and express ciliary projections on the apical surface. The cells also need to perform a variety of passive and active transport. Sodium and potassium are reabsorbed

paracellularly, through ion channels and through transporters such as Na^+,K^+-ATPase and NHE3. Organic solutes such as glucose, amino acids, urea, bicarbonate and phosphate are actively reabsorbed by GLUT9, URAT1, SGLT1/2 and several other sodium dependent co-transporters as well as by endocytosis. Organic ions, including protons and medications such as metformin, lisinopril and others are actively secreted by uptake and efflux transporters such as the organic cation transporters OCT2 and MATE1, the organic anion transporters OAT3 and MRP4 and the non-specific efflux transporter P-gp[7,8]. A complete list of transporters relevant to drug handling is shown in Fig 1.1B.

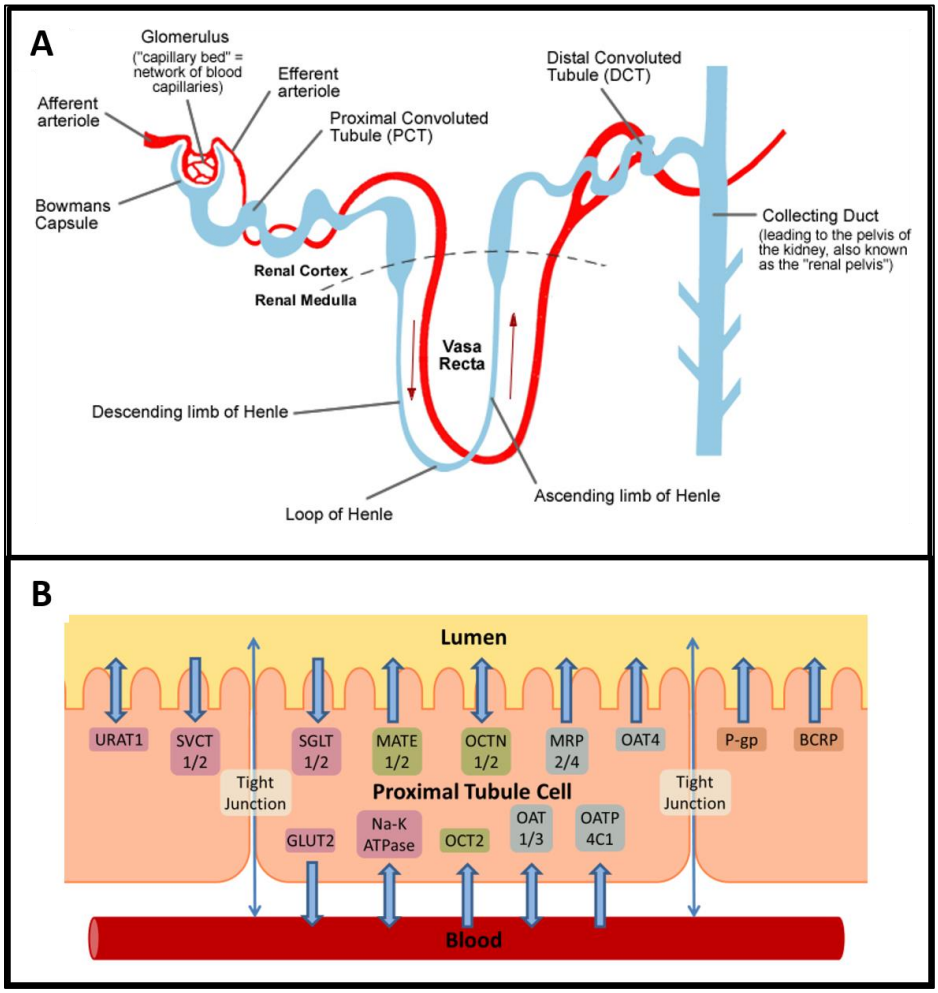


Figure 1.1: Renal Physiology. A) The layout of the nephron and adjacent capillaries[5]. B) The apical membrane contains the urate transporter 1 (URAT1; *SLC22A12*), the sodium dependent vitamin C transporters (SVCT1/2; *SLC23A1/2*), the multidrug and toxin extrusion proteins (MATE1/2; *SLC47A1/2*), the organic cation transporters (OCTN1/2; *SLC22A4/5*), the multi-drug resistance-associated proteins (MRP2/4, P-gp; *ABCC2/4*, *ABCB1*), the organic anion transporter (OAT4; *SLC22A11*), and the breast cancer resistance protein (BCRP; *ABCG2*). The basolateral membrane contains the glucose transporter (GLUT2; *SLC2A2*), the sodium-potassium transporter (Na^+,K^+-ATPase), the organic cation transporter (OCT2; *SLC22A2*), and the organic anion transporters (OAT1/3, OATP4C1; *SLC22A6/8*, *SLCO4C1*).

Cellular Microenvironment:

The *in vivo* environment in the proximal tubule is multifaceted. The tubule itself is a curved monolayer that forms a lumen and is lined by a semi-permeable basement membrane on the basolateral side of the epithelial cells. The basement membrane is composed of a matrix of primarily collagen IV as well as smaller amounts of laminin, entactin/nidogen, and sulfated proteoglycans[9,10]. It provides a porous growth surface and chemical and physical cues that modulate cell behavior. On the other side of the matrix is a bed of capillaries that acts as a basal reservoir of fluid and allows for secretion and reabsorption. The lumen of the tubule is perfused with glomerular filtrate that applies shear stress on the apical side of the cells and along with the capillary fluid, provides an oncotic gradient across the cells. Limited data exist on what an ideal *in vitro* model of the proximal tubule must contain to successfully mimic *in vivo* functionality, but recent work by numerous groups provides some insight[11–20]. Increasing evidence from these studies, described below, has shown that proximal tubule cell morphology and functionality can vary significantly with the growth environment, including growth surface composition and fluid flow.

The structure and components of the surface on which cells are grown may play an important role in the morphology and functionality of cells, and this is an area of active investigation. With respect to structure, it has been shown that cells grown on flat, nonporous surfaces form domes where salts and water accumulate as opposed to a flat monolayer when they are grown on porous surfaces. Furthermore, surface porosity allows for cell polarization and consequently, apical-to-basal and basal-to-apical transport[11,12]. Curvature of the growth surface may also play a potential role in functionality of proximal tubule cells but limited data exist. One recent study looking at the effects of varying levels of curvature on cells found that functionality

of transporters and brush border enzymes was positively correlated with curvature[13]. More work is needed before the role of curvature in renal cell culture models can be clearly defined. With respect to the components of the surface, there is some evidence that the material of the membrane affects cell proliferation rates and adherence, with polysulfone eliciting the best cell behavior[14]. Additionally, studies have shown that collagen IV and/or laminin, when added to porous membranes, significantly improves cell adherence, *in vitro* survival and transport functionality. This has been shown to be further enhanced by double coating with 3,4-dihydroxy-L-phenylalanine[15]. Despite the significant work currently underway into modifying membranes for improved function, there is no clear forerunner, either in terms of underlying materials or coatings and this remains an area of active investigation.

Previous work from Essig and Friedlander and from our lab (Ferrell et al.) demonstrated that tubular fluid flow induced rearrangement in the apical actin cytoskeleton of proximal tubule cells[16,17]. Several studies have also demonstrated that shear stress affects the expression levels and localization of multiple uptake and efflux transporters, including NHE3, Na^+,K^+-ATPase, glucose (SGLT2) transporters and the endocytosis receptors, megalin and cubulin[18–20]. The exact level of shear stress in the human proximal tubule has not been clearly determined. However, based on the glomerular filtration rate of a single nephron calculated from an overall GFR of 60-120 mL/min, the approximate shear stress in the tubules is estimated to be 0.7-1.5 dynes/cm^2 [21]. An ideal device would allow cells to experience a range of shear stresses that encompasses these values and be able to mimic disease states with lower or higher levels. Overall, while there is no conclusive evidence pointing to an ideal microenvironment for proximal tubule cells, it is clear that, at a minimum, a model must incorporate a semipermeable growth surface and shear stress from fluid flow.

Cell Functionality:

Proximal tubule models need to accurately respond to several measures of renal function. The first of these is the uptake and efflux of drug compounds. Drug transport assays are usually performed with fluorescent or radiolabeled compounds wherein cells are exposed to a potential substrate for a defined period of time. Cells must move these substrates across their membranes at levels that can be reliably detected. In addition, they must form a sufficient barrier such that diffusion does not interfere with or confound the measurement of active transport.

Another important function of proximal tubule cells is their response to nephrotoxins. Here, cells are exposed to a potential toxin for a defined period of time and multiple endpoints are measured. General cytotoxicity is measured with a standard live/dead stain, measurement of cell stress by release of LDH or DNA, or an MTT assay that measures cell proliferation/metabolism. In addition, recent research has identified promising biomarkers that are indicators of early kidney injury in the clinic and may have potential in *in vitro* assays as more sensitive markers of proximal tubule cell injury. In response to either toxicity or ischemic injury, renal cells release KIM1, NGAL, NAG1,IL18, L-FABP and GST-α into both the urine and blood[6]. While *in vitro* data regarding these renal biomarkers of early kidney injury are limited, an ideal proximal tubule model would replicate the release of these biomarkers to allow for correlation between *in vitro* and *in vivo* data.

PROXIMAL TUBULE CELLS

A proximal tubule model can use cells from a variety of different sources (Table 1.1). The most biomimetic source of cells would be primary human cells or whole tubules as they are closest to the *in vivo* condition. Primary cells have been shown to retain appropriate morphology and

spontaneously form tubules *in vitro* under certain conditions[22]. They also express functional drug transporters and secrete biomarkers in response to nephrotoxins[22,23]. However, as can be expected, several problems exist with the sourcing and handling of human cells. It is very difficult to obtain healthy cells from human volunteers, particularly when cells from a specific part of the kidney are necessary. Furthermore, once cells are procured, it is very difficult to culture and maintain these cells *in vitro*. Unmodified primary cells quickly lose functionality under standard culture conditions and do not survive multiple passages. Lastly, primary cells suffer from both donor-to-donor variability and contamination by other cell types in the kidney, making them unreliable for screening purposes[24–26].

One way to overcome the issue of limited passage of human primary cells is to use primary animal cells that at least maintain the proximity to *in vivo* conditions. Both primary cells and whole tubules extracted from animals retain proximal tubule characteristics and show drug transport functionality *in vitro*[27]. While this partially alleviates the sourcing problem since it is much easier to isolate cells from animals than from humans, it introduces a different set of issues related to interspecies variability in cell function. Animal proximal tubule cells have different expression levels of transporters and different levels of sensitivity to toxins and therefore are not the most representative model for humans[26]. In addition, primary cells, regardless of source, are still difficult to maintain in culture and donor-to-donor variability remains an issue.

Sourcing and variability issues can be overcome through the use of immortalized cell lines from either humans or animals. Primary cells can be immortalized either spontaneously or by introducing a mutation into the DNA with viral oncogenes such as SV40, allowing the cells to then be passaged indefinitely[28]. A variety of renal cell lines immortalized from rodents, large mammals

and humans exist but cells that have been immortalized by non-spontaneous techniques tend to dedifferentiate and lose functionality after a few passages[29].

Human renal cell lines have been immortalized by both traditional techniques and newer methods of immortalization. Well-established lines are HK-2, a proximal tubule cell line immortalized with HPV16 and HKC-8, a proximal tubule cell line immortalized with a hybrid adeno-12-SV40. While these cell lines retain some functional and morphological characteristics of the renal tubule, neither is able to form a polarized monolayer, which is requisite for accurately testing proximal tubule morphology[30,31]. Newer immortalization techniques have produced more promising cell lines such as HPCTs, which have been immortalized with SV40, RPTECs, which have been immortalized with hTERT and ciPTECs, which have been conditionally immortalized with SV40 such that they proliferate at 33°C but not at 37°C [32–34]. RPTECs transport sodium and glucose, form a polarized monolayer, and express functional organic ion transporters, all indicative of proximal tubule functionality. However, these cells are slow to grow and not amenable to multiple passages[35]. ciPTECs also express transporters and retain many aspects of proximal tubule functionality but no data exist demonstrating their ability to form polarized monolayers[36]. Overall, the newer cell lines show greater promise in accurately representing the proximal tubule but there are still many factors to be investigated before they can be relied upon as accurate models of human renal drug disposition.

The most commonly used models of the kidney are spontaneously immortalized mammalian cell lines: LLC-PK1, a porcine kidney tubular cell line; OK, an opossum kidney cell line; and MDCK I, a canine kidney cortex cell line. Although these cells come with issues of species differences, they form robust monolayers with tight junctions and cuboidal morphology and express several relevant transporters and other proteins, making them a viable option for

modeling the proximal tubule *in vitro*[26,37]. Additionally, these cell lines can be transfected with human transporters, which greatly expands their utility in studying human renal function.

Transfection of transporters into cell lines, both transiently and permanently, is a widely used method of studying renal drug transport. Overexpression by transfection of a single transporter is a robust model for the investigation of the specific role of that transporter in the disposition of a drug and its interaction with inhibitors. Identification of individual transporters involved in drug uptake and/or efflux is important for predicting renal clearance of drugs and accurate prediction of drug-drug interactions in the kidney. More recently, several double transfected models have been generated with one uptake and one efflux transporter inserted (e.g., OCT2/MATE1 and OATP8/MRP2), which allows for the study of the effect of transporter interaction on drug disposition[38,39]. Overall, transfected cell lines do not express transporters at physiological levels and therefore cannot accurately represent drug disposition in the proximal tubule. However, they retain several aspects of functionality *in vitro,* such as monolayer formation, which are unavailable in human cells, and remain robust during long term passaging and culture with low variability, which allows for reproducible testing of specific aspects of transport.

The cell types described above are all mature renal cells that already exhibit a finite amount of functionality. Another possible approach to generating proximal tubule cells is to derive them from stem cells and several groups are working to establish appropriate methods for differentiation. A recent protocol has been shown to successfully differentiate human embryonic stem cells into proximal tubule-like cells that retain a range of functions[40]. Other groups have focused on the generation of whole organoids from renal cells and have shown initial data demonstrating functionality of different parts of the nephron including drug toxicity in the proximal tubule[41,42]. While promising, these protocols for generating renal cells are not established

and have not been shown to be reproducible. Functions related to drug transport and toxicity have also not been thoroughly tested. Further investigations are needed to determine whether stem cells are a viable source of proximal tubule cells. A comprehensive comparison of the various renal cells is also needed to identify the most accurate models of the proximal tubule.

Table 1.1: List of Cell Lines Used for Modeling of Proximal Tubule

Cell Name	Origin	Method of Immortalization	Passages	Morphology	Advantages/ Disadvantages
Primary human cells [22,23]	Human	--	<5	Cuboidal, polarized monolayer, express tight junctions and cilia; Can form tubules *in vitro*	Express drug transporters and metabolic enzymes; secrete biomarkers/ De-differentiate quickly
Primary animal cells [26,27]	Rodent Pig	--	<5	Cuboidal, polarized monolayer, express tight junctions and cilia	Express drug transporters and metabolic enzymes/ Species differences; de-differentiate quickly
HK-2 [30,31]	Human	HPV16	Long term	Cuboidal monolayer	Easy to maintain/ Minimal transporter expression; no tight junctions and leaky monolayer
HKC-8 [30,31]	Human	hybrid adeno-12-SV40	Long term	Cuboidal, polarized monolayer	Easy to maintain/ Minimal functional data
HPCT [32]	Human	SV40	Long term	Non-cuboidal, cancer-like proliferation	Easy to maintain/ Minimal transporter expression; leaky monolayer
RPTEC [33,35]	Human	hTERT	<5	Cuboidal, polarized monolayer, express tight junctions and cilia	Express drug transporters and metabolic enzymes/ Limited passages

Cell Name	Origin	Method of Immortalization	Passages	Morphology	Advantages/ Disadvantages
ciPTEC [20,34]	Human	Conditional SV40 – proliferative at 33°C but not 37°C	5-10	Cuboidal, polarized monolayer, express tight junctions	Express drug transporters and metabolic enzymes/ Leaky monolayer
LLC-PK1 [26,37]	Pig	Spontaneous	Long term	Cuboidal, polarized monolayer, express tight junctions and cilia	Established, can be transfected/Species differences
OK [26,37]	Opossum	Spontaneous	Long term	Cuboidal, polarized monolayer, express tight junctions and cilia	Established, can be transfected/Species differences
MDCK I [26,37]	Dog	Spontaneous	Long term	Cuboidal, polarized monolayer, express tight junctions and cilia	Established, can be transfected/Species differences
Differentiated stem cells [40–42]	Variable	--	Variable	Cuboidal, polarized monolayer, express tight junctions; Can form tubules *in vitro*	Express drug transporters and metabolic enzymes/Early stage so minimal data

SCAFFOLDS TO MIMIC CELL ENVIRONMENT

Static Models:

The most basic environments for modeling the proximal tubule *in vitro* are tissue culture plastic plates and the transwell system, where a porous membrane, usually made of polycarbonate or polyester sulfone, is suspended between two fluidic chambers. These systems are easy to use, commercially available, low cost and compatible with common laboratory equipment. Numerous

studies have been performed on proximal tubule cells grown on these surfaces, both alone and co-cultured with endothelial cells[43,44]. The transwell system is well established and widely used, which allows for easy comparisons of data across studies. However, these systems are minimally physiologically relevant and experiments performed in transwells are unlikely to be fully representative of *in vivo* behavior.

Three-dimensional systems offer greater complexity by allowing cells to be embedded in a more physiologically relevant scaffold. In these cases, cell lines, primary cells and often, whole tubules extracted from tissue or generated *in vitro* are incorporated into the scaffold[45]. The majority of three dimensional scaffolds are typically composed of some combination of collagen I or IV, laminin, fibronectin and/or other extracellular matrix (ECM) proteins. Specific examples of three dimensional systems include ECM-based spheroids that encapsulate cells, a monolayer of cells sandwiched between layers of collagen, and cells or tubules suspended in a Matrigel and/or collagen I matrix[45–47]. These techniques allow better mimicking of *in vivo* conditions and maintain proximal tubule-like characteristics in cells for a longer period of time than 2D cultures. However, because cells are usually embedded in a matrix, manipulation of live cells, imaging and measurement of transport function are very difficult within the 3D systems. A more accessible 3D system is uncoated and ECM-coated polymer hollow fiber tubes that can be seeded with cells[48,49]. The fibers are usually made out of polysulfone or polycarbonate and the cells coat the internal or external surface of the tube, thereby forming a monolayer around a lumen. The fibers can be maintained in media or in a microfluidic system to allow access to nutrients[20,48]. Overall, this system increases accessibility of cells for visualization as well as for uptake and transport assays.

The models mentioned so far are relatively simple and easy to use but only cover a few aspects of renal function. However, the kidney is a complex environment with a variety of

components that are intricately dependent on each other to maintain healthy renal function. To better mimic this interplay, extensive work has gone into generating whole kidney models. One method of doing this is to take an animal kidney, decellularize it such that only the extracellular matrix is retained and then populate it with primary human cells or embryonic stem cells. Decellularized scaffolds retain the architecture of the kidney, ECM based signaling and the renal vasculature, which can be beneficial to the differentiation of cells, the formation of proper monolayers and the testing of transport through the existing fluidic infrastructure[50]. While these scaffolds are promising, it is still very difficult to seed and differentiate cells appropriately in each section of the kidney. A few groups have shown marginal success in murine kidneys but a complete, reproducible method remains to be established[51,52]. Another method for modeling the whole kidney is through direct differentiation of stem cells. There are many ongoing efforts to generate kidney organoids from stem cells and renal progenitor cells, but similarly to decellularized kidneys, much work remains to be done before these organoids can be widely used in the study of renal drug disposition[41,42].

Perfusion Models:

While static culture systems are attractive because they are easy to use and often commercially available, they lack an extremely important component of the *in vivo* environment – shear stress from fluid flow. Traditional cell culture techniques cannot incorporate fluid flow into the model. Therefore, the field is turning to microfluidics and microelectromechanical systems (MEMS) to generate more complex, perfusion based systems. These technologies can generate precisely defined flow paths and pattern microenvironments that will encourage cells to retain functionality.

The most basic perfusion systems are single chamber devices wherein a fluid flow channel is placed over a growth surface on which cells can be seeded. The channel can be connected to a pump and reservoir to allow for constant fluid flow. Most of these devices are made of poly dimethyl siloxane (PDMS) because it allows for rapid prototyping, but other materials can also be used. The growth surface can be a glass slide, PDMS or polystyrene and can be patterned with surface features and/or coated with collagen IV or other ECM matrix components. Surface features can be microstructures to allow for fluid mixing and 3D architecture for cell attachment or micropatterns, such as ridges, that allow for topographic cues and controlled cell adhesion[53,54]. The surface can also be covered with a three-dimensional ECM matrix in which cells can be grown[55]. While these systems were an excellent place to start, they are limited in their ability to mimic the proximal tubule due to the lack of a porous growth surface and access to fluid on both sides of the cell layer and the field has since moved towards more complex models.

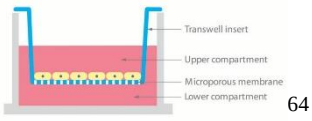
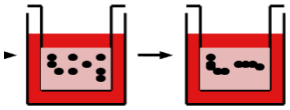
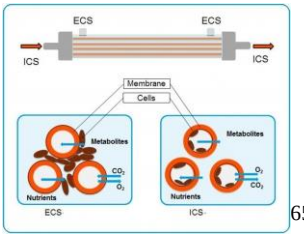
The main type of devices currently being investigated are parallel plate bioreactors composed of two fluidic chambers with a cell growth surface sandwiched in between. The fluidic chambers are usually made from PDMS but can also be made from other polymers or glass. They can be composed of either a single straight channel or have more complex geometry such as serpentine or branched channels to allow for better fluidic mixing or increased surface area or shear stress[56–58]. In some models, fluid is flowed only through the apical channel to mimic urine flow and provide shear stress while the basal channel acts as a static reservoir that simulates interstitial tissue[3,59]. In other models, fluid is flowed through both the apical and the basal channels to simulate urine and blood, respectively[17,58]. All models have a porous growth surface that allows cells to maintain communication between the apical and basal chambers. Some devices incorporate additional features such as continuous TEER measurement[17]. These systems are still being

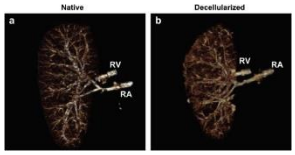
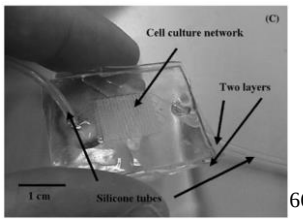
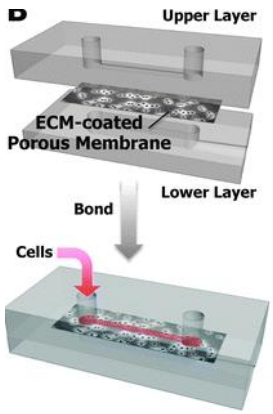
developed and currently are complex and difficult to reproduce. However, they promise to best mimic the proximal tubule of all models to date because they allow cells to polarize, facilitate transport of solutes and water between compartments, and provide shear stress across the apical side of cells.

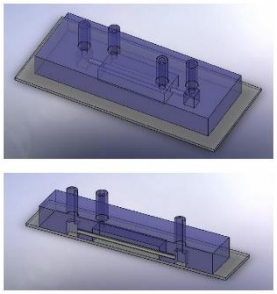
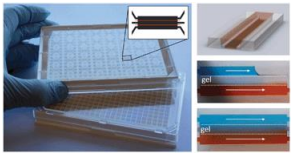
It is uncertain what role curvature may play in supporting proximal tubule cell functionality. Therefore, another approach to two-chamber modeling is to perfuse hollow fiber channels. Several groups have designed microfluidic chips with hollow fiber channels embedded in either PDMS or the more physiologically relevant collagen[60,61]. Cells are usually grown on the inner surface of hollow fibers and fluid is flowed through the lumen. While these devices incorporate the element of curvature, they are limited in the ability to sample basal fluid and to image cells because of the matrix required to support the fibers. Given these issues, parallel plate bioreactors remain the preferred choice for fluidic modeling of the proximal tubule.

While the systems above offer many benefits, they are still limited by the need for external pumping of fluid, are difficult to reproduce and can be bulky. Recently, there has been significant effort to develop models that are high throughput and easy to replicate. One such system is the Organovo plate, which incorporates 96 multi-channel microfluidic perfusion chambers into a single plate[62]. Fluid flow is provided by shaking of the plate rather than a pump and phase guides allow channels to be separated without polymer membranes. This technology is very promising because it allows for reproducibility, co-culturing and integration with existing high-throughput infrastructure but is limited by a lack of control over the shear stress levels to which cells are exposed and the lack of independence in flow rates between different wells. Overall, no perfect system exists for modeling the proximal tubule and additional research is needed to identify important components and incorporate them into a model system.

Table 1.2: Types of Scaffolds Used to Model the Proximal Tubule

Model Type	Description	Examples	Sample Device	Advantages/ Disadvantages
Static Models				
Tissue culture plastic[43,44]	Polystyrene plates	--	 63	Easy to use, commercially available, well established, compatible with lab equipment/ Minimally physiologically relevant
Transwells[4] [3,44]	Porous polycarbonate or polyester sulfone membrane suspended between 2 fluidic chambers	--	 64	Easy to use, commercially available, well established, compatible with lab equipment;/ Minimally physiologically relevant
Three dimensional scaffolds[46]	Typically composed of some combination of collagen I or IV, laminin, fibronectin and/or other ECM proteins	ECM-based spheroids[46]; monolayer sandwiched between collagen layers[47]; cells suspended in matrix[45]		Better mimic of *in vivo* conditions/ Difficult to manipulate and visualize
Hollow fibers[20,48]	Uncoated or ECM-coated polysulfone or polycarbonate hollow fiber tubes seeded with cells	Fibers maintained in media[20]; fibers embedded in microfluidic system[48]	 65	Better mimic of *in vivo* conditions; Better accessibility/Still difficult to manipulate and visualize

Model Type	Description	Examples	Sample Device	Advantages/ Disadvantages
De-cellularized matrix[50]	Animal kidneys with cells removed and repopulated with human cells	--		Retain the architecture of the kidney, ECM based signaling and the renal vasculature/Minimal success - difficult to seed and differentiate cells, difficult to manipulate and visualize
Organoids[41, 42]	Stem cells differentiated into organoids	--		High potential for mimicking renal function/Minimal success
Perfusion Models				
Single chamber[53,54]	Single fluid flow channel placed over a cell growth surface	Fluidic chamber: PDMS or other polymer; Growth surfaces: glass slide, PDMS or polystyrene – plain or patterned	 66	Simple, rapid prototyping, allows fluid flow/Limited mimicking of *in vivo* growth environment
Two chamber - parallel plate[3,17,56–59]	Two fluidic chambers with a cell growth surface sandwiched in between	Fluidic chambers: PDMS, polymer or glass; Geometry: Straight, serpentine or branched; Fluid flow: Apical only or both		Mimics *in vivo* fluid flow and growth surface, allows measurement of transport/ Complex to maintain and reproduce

Model Type	Description	Examples	Sample Device	Advantages/ Disadvantages
Two chamber – hollow fiber[60,61]	Perfused hollow fiber channels	Channels embedded in PDMS[61] or collagen[60]		Mimics *in vivo* fluid flow and growth surface, allows measurement of transport; incorporates curvature/ Limited ability to sample basal fluid and visualize cells
High throughput[62]	Multiple channels that can be simultaneously manipulated	Organovo plate with 96 microfluidic perfusion chambers		Increased reproducibility, potential for co-culture, can integrate with existing high-throughput infrastructure/ Lack of control over shear stress

CONCLUSIONS AND FUTURE DIRECTIONS

Overall, there are two main areas for growth within the kidney-on-a-chip field – broadly, in biology and engineering. With respect to biology, there are many gaps in knowledge about what components of the extracellular environment are important to retain in a proximal tubule model. Much more work needs to be done to systematically assess the various aspects of the *in vivo* environment and identify those that have a direct influence on cell behavior. Additionally, while there are several promising sources of cells available, they have not been well characterized for their ability to mimic the proximal tubule. Further investigations are need to characterize existing cells and generate better sources of cells.

With respect to engineering, the models currently available for use are relatively nascent and there is much room for growth in two potential directions, complexity and throughput, both of which hold value depending on the experimental goals. If the goal is to delve into the biological underpinnings of drug handling and toxicity, current models are primitive, including a small subset of the components of *in vivo* physiology. As our understanding of the proximal tubule extracellular environment expands, there is a need to develop more complex proximal tubule models that incorporate the most pertinent aspects of physiology required for modeling renal drug disposition and toxicity. Moving beyond a singular focus on the proximal tubule, the natural next step is to expand models to add in other components of the kidney such as the glomerulus, the loop of Henle and the distal tubule all of which play significant roles in solute and drug disposition in the human. Lastly, as model complexity increases, it is important to place the kidney within the context of the whole human. This leads to the concept of multi-compartmental models, also known as human-on-a-chip models that can accurately mimic the complex interplay between the various organs of the body and the influence of this cross-talk on drug handling. On the other side, with the accelerated pace of drug discovery and ever increasing volume of potential drug candidates, there is a legitimate need for high throughput models of the proximal tubule. Such a model can sacrifice some physiological complexity in order to allow for rapid testing of toxicity and clearance of novel drug candidates with easy readouts. Both directions are important to pursue to completely address all the needs of the field and it is conceivable that in the future, a model that is both physiologically accurate and high throughput can become the gold standard for drug testing.

SUMMARY OF CHAPTERS

The focus of this dissertation is to address three of the gaps in knowledge discussed above. In the second chapter, multiple proximal tubule cell lines are systematically compared in order to

identify the most physiologically relevant one. In the third chapter, the role of one particular aspect of the *in vivo* environment, namely shear stress, is studied to better understand the effect on drug transporters and the possible biological pathways involved. In the fourth chapter, the design of a more complex, next generation model of the kidney is proposed and prototyped. A brief summary of each of these chapters is provided below.

Chapter 2: Characterization of Proximal Tubule Cell Lines for Morphology and Organic Cation Transporter Functionality

The proximal tubule is the most important part of the kidney to model for drug clearance and toxicity studies. While several cellular models exist, few studies have systematically compared different cell types across relevant metrics. In this study, we compare three cell lines (MDCKs transfected with a pair of human organic cation transporters (hOCT2/hMATE1), SV40 immortalized human proximal tubule cells (HPCT) and hTERT immortalized human proximal tubule cells (RPTEC)) across a range of functions including morphology and monolayer formation, gene expression, organic ion transport and response to a nephrotoxin. While RPTECs are superior in terms of morphologically mimicking the proximal tubule, they show high variability in active organic cation transport when compared to the transfected MDCKs. The transfected MDCK cells are also better at mimicking *in vivo* response to cisplatin in terms of cell viability, biomarker release and mitigated toxicity with uptake inhibitors. Overall, while the human cell lines show promise, the double transfected canine cells remain the more robust, albeit limited, model of the human proximal tubule.

Chapter 3: Apical Shear Stress Enhanced Organic Cation Transport in hOCT2/hMATE1 Transfected MDCK Cells Involves Ciliary Sensing

Active transport by renal proximal tubules plays a significant role in human drug disposition and is therefore important to investigate when developing drugs. Kidney bioreactors that reproduce physiological cues in the kidney, such as flow-induced shear stress, may better predict *in vivo* drug behavior than current *in vitro* models. In this study, the effect of graded levels of shear stress on active transport of ASP+ was investigated using a parallel plate bioreactor cultured with Madin-Darby Canine Kidney (MDCK) cells exogenously expressing the human organic cation transporters OCT2 and MATE1. Cells placed under flow formed a tight monolayer with high barrier to inulin. In response to increasing levels of shear stress (0.2-2 dynes/cm^2), cells showed a corresponding increase in transport of ASP+, an organic cation, reaching a maximal 4.2-fold increase at 2 dynes/cm^2 when compared to cells cultured under static conditions. This transport was inhibitable with imipramine, indicating active transport was present under shear stress conditions. Cells exposed to shear stress of 2 dynes/cm^2 also showed an increase in RNA expression of both transfected human and endogenous OCT2 (3.7- and 2.0-fold, respectively). Lastly, the role of cilia in this shear stress response was investigated by measuring transport after cilia removal by ammonium sulfate. Removal of cilia eliminated the effects of shear on ASP+ transport at 0.5 dynes/cm^2 with no effect on ASP+ transport under static conditions. These results indicate that shear stress affects active transport of organic cations and expression of relevant transporters in renal cells in a cilia dependent manner.

Chapter 4: Design of a Scaled Model of the Glomerulus and Proximal Tubule

Disposition of novel drugs in the body is currently determined mainly through animal testing before entering clinical trials. However, animals are poor mimics of drug disposition and

one way to overcome this is to develop *in vitro* human-on-a-chip models that are functionally scaled to mimic inter-organ interaction in the human body. Here, the design of a 1:1000 scaled model of the glomerulus and proximal tubule is presented along with scaling metrics and design parameters. Preliminary results from initial prototyping are presented and future directions for such a model are discussed.

Chapter 5: Conclusions and Future Directions

In this chapter, the major findings of the dissertation are summarized and future directions are suggested for better understanding the role of the microenvironment on proximal tubule cell functionality and for the generation of more physiologically accurate models of the tubule.

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CHAPTER 2:

CHARACTERIZATION OF PROXIMAL TUBULE CELL LINES FOR MORPHOLOGY AND ORGANIC CATION TRANSPORTER FUNCTIONALITY

INTRODUCTION

The proximal tubule of the kidney is responsible for reabsorption of nutrients and secretion of toxins and xenobiotics. Salts such as sodium and potassium are mostly reabsorbed paracellularly while organic solutes such as glucose and amino acids, urea and phosphate are actively reabsorbed. Protons and organic ions such as metformin, lisinopril, and other small molecules are actively secreted by uptake and efflux transporters in the tubule. Within the nephron, the proximal tubule handles the largest quantity of drug transport and is therefore important for understanding drug clearance and nephrotoxicity[1,2].

Transporters of the proximal tubule play an integral role in *in vitro* testing of new drugs[1]. While primary human cells would be ideal to test drug disposition in the kidney, they are difficult to isolate and lose functionality in culture beyond a few passages[3]. To overcome this limitation and to identify an appropriate model of the human proximal tubule, multiple cell lines have been developed in the past few decades using various immortalization methods on proximal tubule cells isolated from multiple species. Much of the current work is performed in the well-established porcine LLC-PK1 and the canine MDCK cells[3]. In many cases, these cell lines are transfected with human transporters. While useful, these models are limited in their ability to comprehensively represent all aspects of human proximal tubule function. More recently, efforts have been directed

at developing improved immortalized human cell lines, including SV40, hTERT, and conditionally immortalized lines[4–6]. While these cells are promising, little data exist comparing their functionality. A systematic comparison of the cell lines across a range of metrics relevant to proximal tubule functionality would move us substantially closer to identifying or generating an ideal model. Such models could be used for not only drug testing but more broadly in organ-on-a-chip and even implantable kidney applications.

In this study, MDCK cells transfected with a pair of human organic cation transporters (hOCT2/hMATE1)[7], SV40 immortalized human proximal tubule cells[4] and hTERT immortalized human proximal tubule cells[5] are evaluated. A range of functions including morphology and monolayer formation, gene expression, organic ion transport and response to a nephrotoxin are compared to determine the most appropriate model for studying human renal drug disposition.

METHODS

Cell Culture:

MDCK cells transfected with either an empty vector or a pair of uptake and efflux transporters (hOCT2/hMATE1) were kindly provided by Dr. Martin Fromm (Stuttgart, Germany) and were cultured in MEM with Earle's BSS (UCSF Cell Culture Facility) with 10% FBS (Gibco, Thermo Fisher Scientific) and 1% penicillin-streptomycin (UCSF Cell Culture Facility); hygromycin (UCSF Cell Culture Facility) and geneticin (Sigma-Aldrich) were added to the media for the dual transfected cells. Cells were typically cultured for 4-7 days on transwells (Corning) until confluency was reached.

HPCT (Human proximal convoluted tubule) 9405 cells immortalized with SV40 were kindly provided by Dr. Ulrich Hopfer (Case Western University) and were cultured in DMEM/Ham's F12 (Sigma-Aldrich) with 5% FBS (Gibco, Thermo Fisher Scientific), 1% penicillin-streptomycin

(UCSF Cell Culture Facility), glucose, transferrin, insulin, epidermal growth factor, dexamethasone, HEPES, $NaHCO_3$, L-glutamine, ascorbic acid 2-phosphate, sodium selenite and 3,3',5-triiodo-L-thryonine (all Sigma-Aldrich) as described in a previous publication[4] . Cells were cultured for 7-10 days on transwells until confluency was reached.

RPTEC cells (renal proximal tubule epithelial cells) immortalized with hTERT were purchased from ATCC and cultured in DMEM/Ham's F12 (Sigma-Aldrich) with geneticin, L-glutamine, HEPES, 3,3',5-triiodo-L-thryonine, recombinant human EGF, ascorbic acid, transferrin, insulin, prostaglandin E_1, hydrocortisone and sodium selenite (all from Sigma-Aldrich) as described by ATCC. Cells were cultured for 10-14 days on transwells until confluency was reached.

Immunofluorescence:

Cells were fixed with 4% paraformaldehyde (Pierce, Thermo Fisher Scientific), permeabilized with 0.1% Triton-X buffer (Sigma-Aldrich) and blocked with BSA (Sigma-Aldrich). They were subsequently incubated with a 1:50 dilution of Alexa 488-labeled zonula occludens (ZO-1) mouse monoclonal antibody (Life Technologies) or a primary acetylated α-tubulin mouse monoclonal antibody (Life Technologies) for 60 minutes. Cells treated with the α-tubulin antibody were then incubated with a secondary 488 or 561 anti-mouse goat antibody (Life Technologies). Imaging was performed using a Nikon spectral confocal microscope with a 40X oil objective.

Inulin Permeability:

Radiolabeled inulin (Perkin Elmer) was added to the basal compartment and measured in the apical compartment after one hour. Inulin content was measured using a scintillation counter (Beckman).

RNA Expression:

RNA was extracted from cells using an RNEasy RNA Extraction Kit (Qiagen) and cDNA was generated using an iScript kit (Bio-Rad). cDNA was used for detection of human and dog OCT2,

MATE1 and P-gp, human OAT3 and MRP4 by qRT-PCR using a Taqman assay and probes (Applied Biosystems) on the Fast Realtime PCR instrument (Applied Biosystems). Dog RS-18 or human GAPDH expression were similarly quantified as controls and transporter levels were expressed relative to the control gene (ΔCt)[8].

Organic Cation Transport:

MDCK cells only were treated with 10 mM sodium butyrate (Sigma-Aldrich) 24 hours prior to a transport experiment, as described previously[7] . For transport, cells were incubated with an inhibitor (500 μM imipramine or 1 mM cimetidine (Sigma-Aldrich)) on the basal side for 30 minutes, followed by addition of either 25 μM 4-(4-dimethylamino)styryl-N-methylpyridinium (ASP+) (Life Technologies) or 500 μM metformin (Sigma-Aldrich) (1:2000 ^{3}H-radiolabeled compound (American Radiolabeled Chemicals)) and incubated for 1 hour. Samples were collected from the apical and basal media and cells were lysed for measurement of ASP+ or metformin accumulation and transport. ASP+ was quantified on a Genios Pro fluorescence plate reader (Tecan) at an excitation wavelength of 485 nm and an emission wavelength of 590 nm. Metformin was quantified using a scintillation counter (Beckman).

Protein Quantification:

Cells were lysed with 1% SDS-10 M NaOH lysis buffer while shaking overnight. Protein content was measured using a standard Pierce BCA protein assay kit (Thermo Fisher).

Cisplatin Toxicity:

MDCK cells were treated with 10 mM sodium butyrate 24 hours prior to a transport experiment, as described previously[7]**.** Nephrotoxicity was evaluated in cells incubated with or without an inhibitor (1 mM cimetidine) and 0, 10 or 100 μM cisplatin (Sigma-Aldrich) for 24 hours. Samples were collected from apical and basal media and cells were incubated with CellTox Green

cytotoxicity reagents (Promega) to measure toxicity on a Genios Pro fluorescence plate reader (Tecan). Expression of KIM1 was detected with the canine KIM1 (ab205084) ELISA kit (Abcam) or the human KIM1/TIM1/HAVCR (EHHAVCR1) ELISA kit (Thermo Fisher Scientific). Expression of NGAL was detected with the human (ab113326) ELISA kit (Abcam) or dog (ab205085) NGAL ELISA kit (Abcam).

Statistics:

Most experiments were performed in triplicate with a minimum of two technical replicates within each experiment. Cisplatin toxicity experiments in the RPTEC cells were performed in duplicate. Data is expressed as mean ± standard deviation and graphed as box (median and 25^{th}-75^{th} quartiles) and whisker (min/max) plots. Statistical analyses were performed by unpaired one way or two way Anova and a p value of <0.05 was considered significant. Data were analyzed using Prism Version 6.0 (Graphpad).

RESULTS

Cell Characterization:

Three different cell lines were compared on a variety of metrics to probe their ability to mimic renal proximal tubule function. First, morphology was investigated by staining for tight junctions and cilia. Both MDCK cells and RPTEC cells expressed tight junctions but RPTEC cells were much less densely packed than MDCK cells (Fig. 2.1A and 2.1E). HPCT cells also expressed tight junctions, but unlike the other two cell lines, grew on top of each other in layers resulting in an uneven monolayer (Fig. 2.1C). Cilia were visible on both MDCK cells and RPTEC cells but not the HPCT cells (Fig 2.1B, 2.1D and 2.1F). MDCK cells expressed shorter cilia while RPTEC cells expressed longer, slightly bent cilia. As determined by tight junction formation and inulin permeability measurements, MDCK cells reach confluency after 4 days, HPCT cells after 7 days,

and RPTEC cells after 7-10 days. At confluency, MDCK cells showed the lowest inulin permeability at 1.5 ± 0.1% over 1 hr; RPTEC cells had 2.5 ± 0.08%, and HPCT cells 7.3 ± 0.4% inulin permeability over this time period (Fig 2.1G).

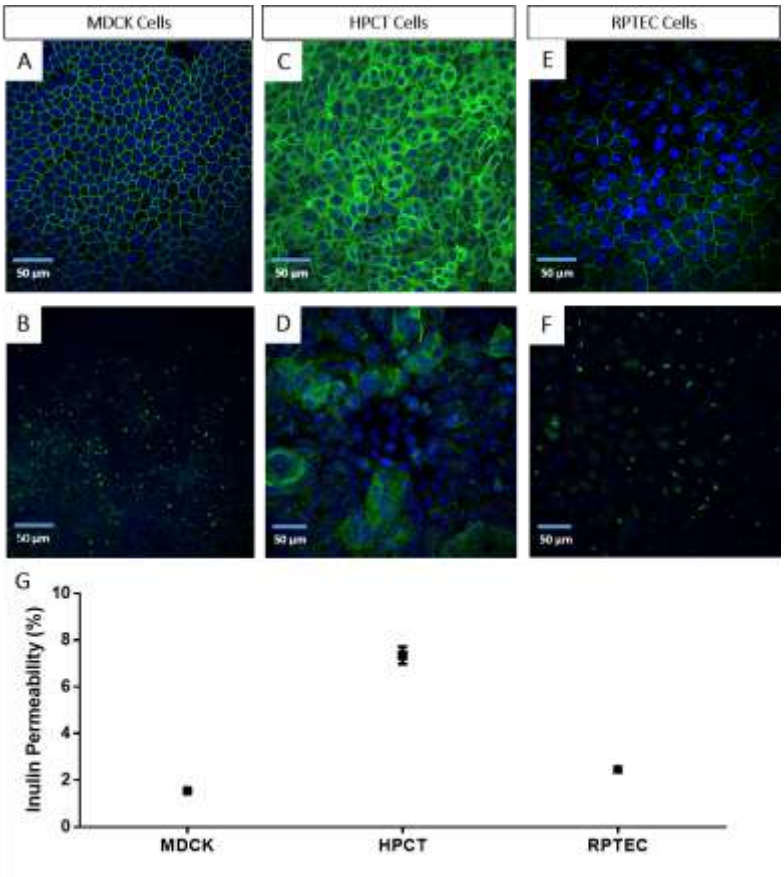

Figure 2.1. Morphology of Renal Proximal Tubule Cells. A-F) Three cell lines (hOCT2/hMATE1 double transfected MDCK, HPCT and RPTEC) were grown to confluency on transwells and then stained with either α-tubulin for cilia (A,C,E) or ZO-1 for tight junctions (B,D,F) as well as DAPI for the nucleus; G) Cell lines were grown to confluency on transwells and then inulin was added basally and permeability across the cells was measured over the course of one hour. Experiments were performed in triplicate and data are expressed as percent (±SD) of the donor amount of inulin that leaked into the apical compartment.

RNA expression was quantified to determine expression levels of several cationic and anionic renal drug transporters. As expected, the transfected MDCK cells expressed the highest levels of human OCT2 and MATE1 but also expressed dog OCT2 at lower levels. Both HPCT and RPTEC cells expressed similar levels of OCT2 and MATE1 (Fig 2.2) as well as OAT3 and MRP4 with RPTEC cells expressing slightly higher levels of MRP4 (data not shown).

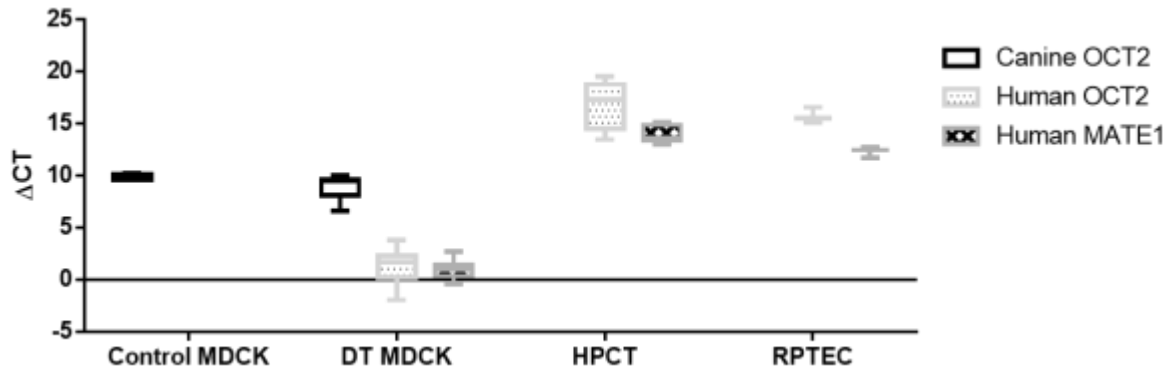

Figure 2.2. RNA Expression in Cell Lines. Cell lines were grown to confluency and RNA expression of dog OCT2, human OCT2, and human MATE1 was measured. Data are expressed as change in cycle threshold over the housekeeping gene (RS-18 for the MDCK cells and GAPDH for the human cells) with smaller numbers reflecting higher expression.

Organic Cation Transport:

To test functionality of OCT2 and MATE1, the uptake and transport of ASP+ and metformin, both organic cations, were investigated. The double transfected MDCK cells accumulate ASP+ at 1.30-33.5 µmol/mg protein/hr and transcellular transport of ASP+ was 8.40-141 µmol/mg protein/hr. Accumulation and transport in the MDCK cells were significantly inhibited by cimetidine 48.8 ± 15.4% and 83.0 ± 10.8%, respectively. While absolute accumulation

and transport rates varied considerably across experiments, the percent relative inhibition reflecting transporter-mediated movement was relatively constant. Accumulation and transport rates for ASP+ in HPCT cells were 1.62 ± 0.55 µmol/mg protein/hr and 16.2 ± 7.8 µmol/mg protein/hr, respectively. In contrast to the MDCK cells, neither accumulation nor transport of ASP+ in HPCT cells was significantly inhibited by cimetidine (3.01 ± 3.59% and 6.40 ± 8.84%, respectively), indicating no active transport. RPTEC cells accumulate ASP+ at 6.00 ± 1.82 µmol/mg protein/hr and corresponding transport rates were 3.90 ± 3.52 µmol/mg protein/hr. ASP+ accumulation and transport in the RPTEC cells were significantly inhibited by imipramine 65.1 ± 12.2% and 28.5 ± 4.1%, respectively (Fig 2.3).

Metformin transport was also measured and showed similar trends as ASP+ transport. The double transfected MDCK cells accumulate metformin at 0.732 ± 0.309 µmol/mg protein/hr and transport metformin across the cell at 113 ± 31 µmol/mg protein/hr, and both were significantly inhibited by cimetidine (61.3 ± 16.3% and 78.8 ± 6.3%, respectively). Metformin accumulation rates in HPCT cells were 0.368 ± 0.177 µmol/mg protein/hr and transcellular transport rates were 67.1 ± 29.1 µmol/mg protein/hr. Neither rates were significantly inhibited by the addition of cimetidine.

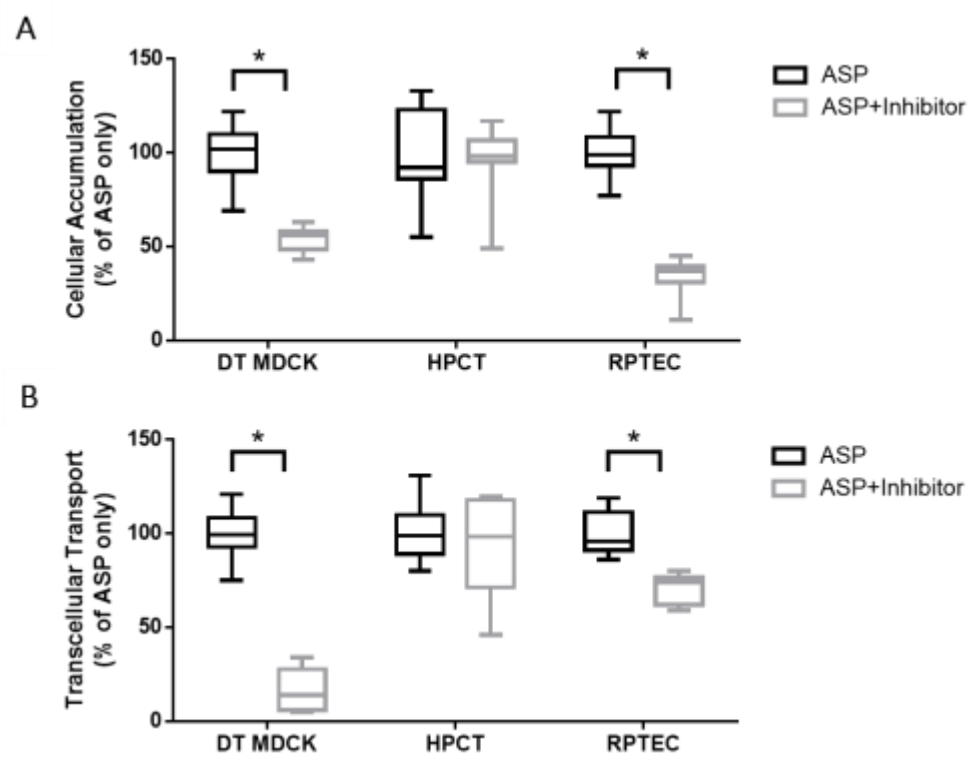

Figure 2.3. ASP+ Transport by Proximal Tubule Cell Lines. A-B) Cells were grown to confluency on transwells, pre-treated basally with an inhibitor (either imipramine or cimetidine) for 30 minutes where indicated and then exposed to ASP+ on the basal side (with or without inhibitor) for one hour. Accumulation into cells (A) and transcellular transport (B) of ASP+ were measured and expressed here as percent of uninhibited transport within each cell line. Experiments were performed in triplicate and data are shown as box (median with 25^{th} to 75^{th} percentile) and whiskers (min and max) plots; * indicates $p<0.05$.

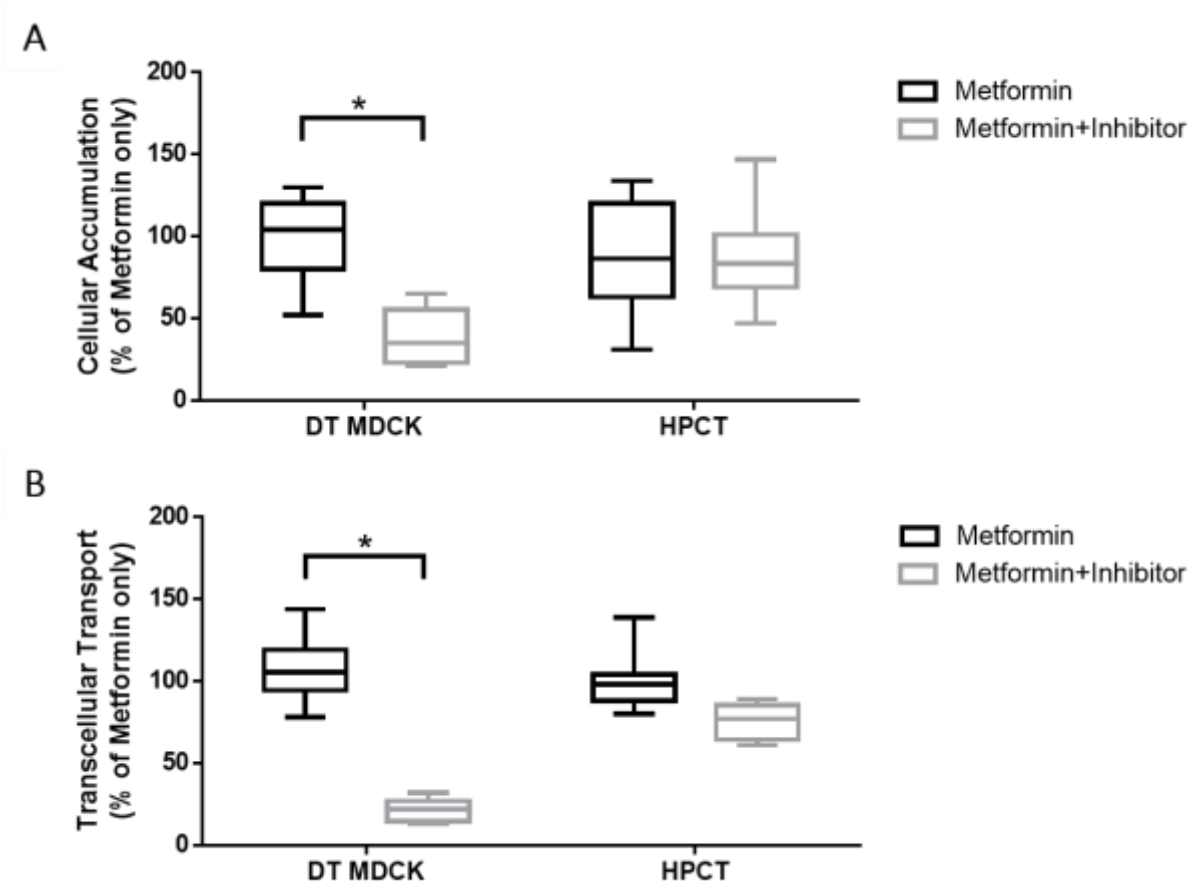

Figure 2.4. Metformin Transport by Proximal Tubule Cell Lines. A-B) Cells were grown to confluency on transwells, pre-treated basally with an inhibitor (cimetidine or imipramine) for 30 minutes where indicated and then exposed to metformin on the basal side (with or without inhibitor) for one hour. Accumulation into cells (A) and transcellular transport (B) of metformin were measured and expressed as percent of uninhibited transport. Experiments were performed in triplicate and data are shown as box (median with 25^{th} to 75^{th} percentile) and whiskers (min and max) plots; * indicates $p<0.05$.

Cisplatin Toxicity

The responsiveness of the cells to a known nephrotoxin, cisplatin, was measured after 24 hour exposure to 10 or 100 µM. Similar trends in cell viability were observed in all three cell lines. Double transfected MDCK cells showed increasing cell death in response to increasing cisplatin concentrations; cells exposed to 10 µM and 100 µM cisplatin had 1.82 ± 0.79 and 3.25 ± 1.40 times as much cell death as the untreated control, respectively. Toxicity from 10 µM and 100 µM cisplatin was inhibited 53.3 ± 18.3% and 43.6 ± 28.1%, respectively by cimetidine and was statistically significant at the higher concentration (Fig 2.5A). HPCT cells also showed dose-dependent toxicity to cisplatin resulting in 1.57 ± 0.65 times and 4.05 ± 2.91 times as much cell death as untreated controls at 10 and 100 µM cisplatin, respectively. Cell death in the HPCT cells was not significantly inhibited by cimetidine (Fig 2.5B). Cisplatin was minimally toxic to RPTEC cells, even at the higher concentration of 100 µM (Fig 2.5C).

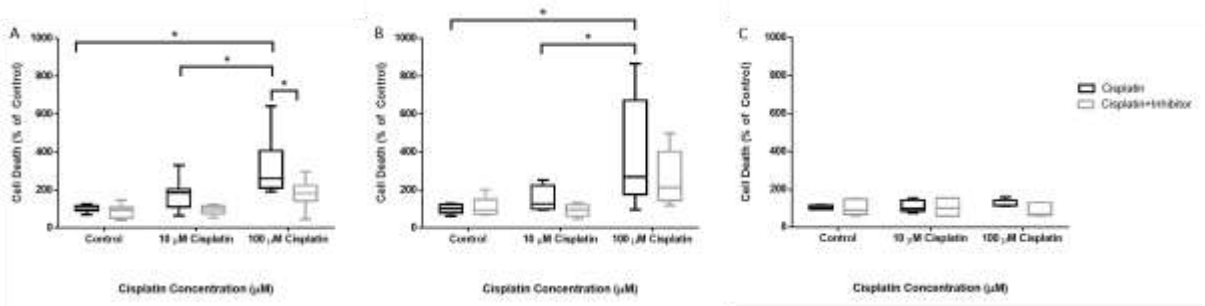

Figure 2.5. Cell Death After Cisplatin Exposure. A) MDCK, B) HPCT and C) RPTEC cells were grown to confluency on transwells and exposed to 0-100 µM cisplatin with or without inhibitor (cimetidine) as indicated for 24 hours. Cell death was measured with a cytotoxicity assay and expressed relative to untreated cells. Experiments were performed in triplicate and data are shown as box (median with 25th to 75th percentile) and whiskers (min and max) plots; * indicates $p<0.05$.

Release of KIM1 and NGAL, two biomarkers of acute kidney injury, was measured after 24 hours of exposure to cisplatin. Double transfected MDCK cells secrete KIM1 at a baseline level of around 263 ± 77.5 μg/cm^2 and this was not affected by exposure to cisplatin (Fig 2.6A). RPTEC cells secrete KIM1 at a baseline level of 94.4 ± 36.2 μg/cm^2 and this level was reduced to 56.6 ± 13.6 μg/cm^2 after 24 hour exposure to cisplatin (Fig 2.6B). HPCT cells do not secrete KIM1 at detectable levels. MDCK cells secrete NGAL at a baseline level of 1.35 ± 0.49 μg/cm^2 and the secretion unexpectedly decreased to 0.419 ± 0.172 μg/cm^2 when cells were exposed to 100 μM cisplatin. This effect was mitigated when cimetidine was added concurrently with the cisplatin such that the decrease was only 24.7 ± 20.4% compared to 67.4 ± 10.2% in the absence of cimetidine (Fig 2.6C). HPCT cells showed similar trends with a baseline expression of NGAL at 17.5 ± 2.9 μg/cm^2 which decreased by 78.5 ± 3.5% to 3.96 ± 0.62 μg/cm^2 after 24 hour cisplatin exposure (Fig 2.6D). RPTEC cells also showed similar trends with a baseline expression of NGAL of 0.0837 ± 0.0444 μg/cm^2, which decreased by 35.6 ± 4.2% to 0.0531 ± 0.0267 μg/cm^2 after 24 hour cisplatin exposure. Cimetidine had no effect on NGAL levels in either human cell line (Fig 2.6E).

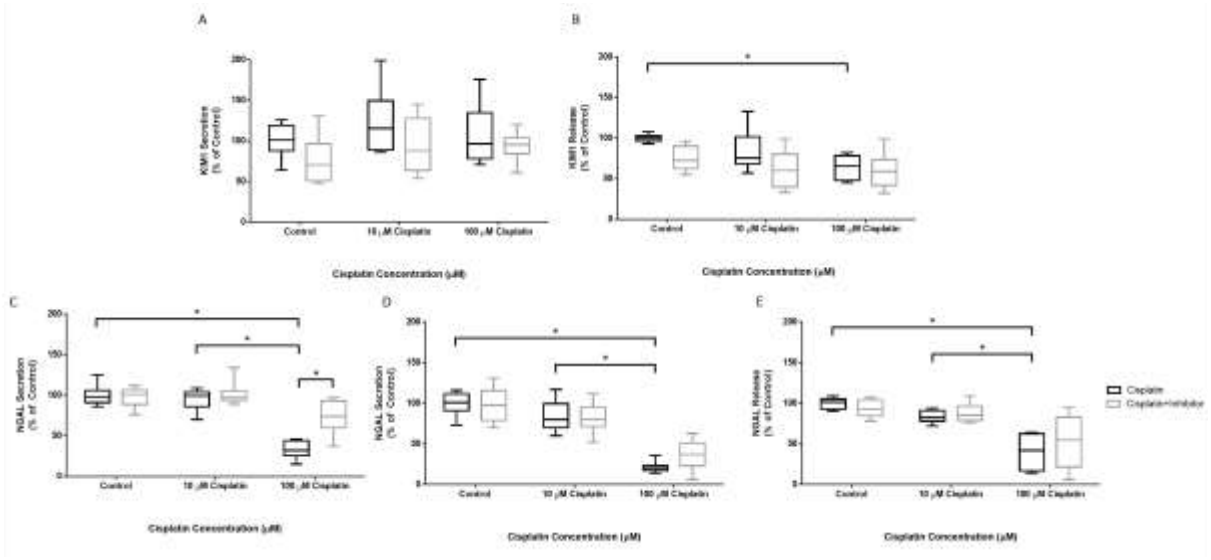

Figure 2.6. Cellular Response to Cisplatin Exposure. Cells were grown to confluency on transwells and exposed to 0-100 µM cisplatin with or without inhibitor (cimetidine) as indicated for 24 hours. Secretion of KIM1 in MDCK (A) and RPTEC cells (B) and NGAL in MDCK (C), HPCT (D) and RPTEC (E) cells was measured and expressed as percent of release by untreated cells. HPCT cells did not release detectable levels of KIM1. Experiments were performed in triplicate and data are shown as box (median with 25th to 75th percentile) and whiskers (min and max) plots; * indicates $p<0.05$.

DISCUSSION

The proximal tubule is responsible for most drug disposition in the kidney and is therefore important to model when testing drug clearance and toxicity[1]. A variety of cell lines exist, including recently immortalized human cells, but few comparative studies have been performed across these models[4,5,7]. In this study, three different cell lines, MDCK cells transfected with a pair

of human organic cation transporters (hOCT2/hMATE1), SV40 immortalized human proximal tubule cells and hTERT immortalized human proximal tubule cells, were assessed using a range of proximal tubule functionality metrics. Specifically, metrics relevant to renal drug handling were selected, including morphology and monolayer formation, gene expression and functionality of organic cation drug transporters, and cell viability and biomarker release after exposure to cisplatin.

These studies demonstrate that RPTEC cells most closely resemble proximal tubule cell morphology among the three cells lines tested. RPTE and MDCK cells both displayed a cuboidal cell shape with distinct tight junctions lined by zona-occludens 1. Additionally, both cell lines display cilia but RPTEC cells have longer, bent cilia more characteristic of proximal tubule cells. This morphology aligns closely with both primary proximal tubule cells and proximal tubule tissue sections[9,10]. Unlike the first two cell lines, HPCT cells were unable to maintain cuboidal morphology or produce cilia. Furthermore, while all cells had an inulin permeability below 10%, a reasonable threshold for acceptable monolayer tightness, HPCT cells were much leakier than MDCK cells and RPTEC cells, both of which had similar inulin permeability levels. Leaky monolayers make it difficult to differentiate active transport from paracellular diffusion, and therefore, HPCT leakiness puts them at a significant disadvantage over the other two cell lines when investigating the contribution of membrane transporters in drug disposition and toxicity.

Double transfected MDCK cells are more reliable when it comes to drug transporter expression and function. RPTEC cells and HPCT cells both express drug transporters but, as expected, levels are much lower than the overexpressing MDCK cells. This also affected functionality, as demonstrated with two organic cation substrates. ASP+ has a high affinity for OCT2 with a K_m of approximately 25 µM[11]. Metformin has a lower affinity with a K_m of

approximately 6800 μM[12]. While both human cell lines transport ASP+, the levels of both overall and active transport are much lower than the transfected MDCK cells. In addition, the HPCT cells show no evidence of active metformin transport as reflected by insensitivity to cimetidine. In contrast, the transfected MDCK cells demonstrate transporter specific transport of both organic cations. The inability of the human cells to transport all organic cations, especially lower affinity transporter substrates, significantly limits their usefulness as a model of renal drug transport. Based on the functional data presented here, the transfected MDCK cells are clearly the more robust model for measuring organic cation transport. It is important to note, however, that the transfected MDCK cells had high inter-experimental variability in the absolute accumulation and transport values that could not be directly linked to passage number. Relative inhibition of organic cation transport remained consistent across experiments, which allows for reliable monitoring of active transport. Whether the variability in absolute transport rates may limit the range of applications for the MDCK cells requires further investigation.

Lastly, response to cisplatin exposure was considered, and although the results were complex, they support the conclusion that MDCK cells are the best system for predicting nephrotoxicity. MDCK and HPCT cells showed reduced viability in response to cisplatin exposure, but this was attenuated by cimetidine, an inhibitor of organic cation transport, only in the MDCK cells. Interestingly, RPTEC cells were much less sensitive to cisplatin and showed no significant cell death in response to exposure. It will be of interest to identify the factors that confer resistance of the RPTEC cells to cisplatin.

NGAL and KIM1, two established markers of early kidney injury, are upregulated in response to cisplatin toxicity in animal and human samples[13]. In this study, KIM1 was detected in MDCK cells and RPTEC cells but was modestly affected by cisplatin only in RPTEC cells. All

three cell lines had reduced NGAL secretion in response to 100 µM cisplatin but only in the MDCK cells was this attenuation attributed to organic cation transport based on sensitivity to cimetidine. Based on *in vivo* experiments, the levels of NGAL and KIM1 were expected to be upregulated in response to cisplatin toxicity[14,15], which is contrary to the data presented here. Relatively few *in vitro* studies have measured the effect of nephrotoxins on these biomarkers; typically RNA expression changes in KIM1 and NGAL are measured with the former consistently increased and the latter having variable results[16,17]. *In* vitro studies measuring KIM1 release by cells have typically found no changes in secretion of the protein in response to cisplatin[17,18]. The unexpected but modest decrease found in RPTEC cells in this study is likely a nonspecific change given the lack of sensitivity to cisplatin. One *in vitro* study measured NGAL release into supernatant by primary proximal tubule cells exposed to cisplatin over the course of weeks and found an increase in NGAL secretion but more acute changes in secretion were not reported[19]. In another study in HK-2 cells, NGAL release was inversely correlated with cell toxicity within the first 24 hours following cisplatin treatment[20]. These discordant results between *in vitro* and i*n vivo* release in response to cisplatin, particularly of NGAL, do not support a role for these human biomarkers of renal injury in cellular studies.

The goal of this study was to compare functionality of immortalized human proximal tubule cells against the current standard, transfected MDCK cells. Human cells would be ideal since the species relevance and endogenous nature of the transporters would allow drug testing to be influenced by the full spectrum of biological activity relevant to drug handling. Canine cells, especially transfected ones, do not accurately reflect drug transport by human proximal tubule cells. Given the data presented here, however, the double transfected cells remain the most reliable model for the proximal tubule. While at least one of the human cell lines is morphologically

superior, transporter activity is one of the most crucial aspects of drug testing and inconsistencies in handling of organic cation substrates is a major roadblock to the reliable use of human cells. Of course, the canine cells tested here also have limitations, particularly with respect to variability in absolute transport rates, the number of transporters that can be tested and the background activity of endogenous canine transporters. Furthermore, the study itself is limited as many other biological functions including transport of endogenous substrates and organic anions were not tested. Since transfection improves the measurement of drug transport, efforts should be made to develop RPTEC cells overexpressing relevant membrane transporters in order to benefit from both accurate morphology and robust transporter activity.

CONCLUSION

In summary, the data presented here demonstrate that while human derived cell lines hold promise, non-human transfected cell lines remain more robust models of proximal tubule drug transport. While further study is required to understand biomarker release in response to cisplatin toxicity, these data also supports the conclusion that MDCK cells are a superior model. Overall, this study demonstrates the need to systematically compare new cellular models with existing ones across a broad range of metrics in order to properly understand their usefulness in characterizing renal drug disposition and toxicity.

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CHAPTER 3:

APICAL SHEAR STRESS ENHANCED ORGANIC CATION TRANSPORT IN hOCT2/hMATE1 TRANSFECTED MDCK CELLS INVOLVES CILIARY SENSING

INTRODUCTION

Despite major advancements in preclinical methods for selection of optimal drug candidates, drug development remains a long and expensive process that has a very low yield of marketed new molecular entities[1]. A major shortfall of preclinical models is the inability to accurately predict clearance and toxicity in humans. Approximately 30% of drugs that are successful in preclinical studies fail in humans due to unanticipated clearance or toxicity differences.[2]

The kidney is a particularly important target for *in vitro* cell culture modeling because it is responsible for elimination of over one third of all drugs and a majority of metabolites[3]. The proximal tubule of the kidney is of primary importance for drug testing during development because it performs the majority of active transport of drug candidates and is particularly sensitive to toxic injury[4]. The tubule is a monolayer of epithelial cells with filtrate flowing across the apical surface. A basement membrane underlies the tubular epithelium and adjacent peritubular capillaries allow for reabsorption of water and solutes. The environment experienced by proximal tubular cells is quite complex with constant exposure to fluid shear stress and apicobasal oncotic gradients. Cell culture on impermeable substrates such as tissue culture plastic incompletely replicates this environment. Epithelial cell bioreactors that capture salient *in vivo* physiology may improve accuracy of clearance and toxicity predictions derived from *in vitro* assays[5,6].

There is growing evidence that proximal tubule cell morphology and functionality can vary with the growth environment including growth surface porosity, exposure to fluid on both sides and/or fluid shear stress. Previous work from Essig and Friedlander and from our lab (Ferrell et al.) demonstrated that tubular fluid flow induced rearrangement in the apical actin cytoskeleton of proximal tubule cells[7,8]. Subsequently, several studies demonstrated that shear stress affects the expression levels and localization of multiple uptake and efflux transporters, including NH3, Na^+/K^+-ATPase, glucose (SGLT2) transporters and the endocytosis receptors, megalin and cubulin[9–11]. Recent work also indicates that some of the alterations in cytoskeletal structure and transport function are likely related to the mechanosensory function of cilia[10,12]. However, few studies have considered the effect of shear stress on drug transporters in renal cells. Furthermore, little is known about the effect of different shear rates on cell functionality, particularly under sustained shear stress exposure. Most studies have considered a single shear stress rate and have only performed short term experiments (1-6 hours), making it difficult to distinguish between the true effects of shear and the cellular stress response to abrupt changes to the microenvironment[9,13,14].

Here, a microfluidic bioreactor was used to examine the effect of graded levels of shear stress on the renal proximal tubule cell drug transporters OCT2 and MATE1. We demonstrate that increasing apical shear stress leads to increasing organic cation transport and transporter expression and that cilia are involved in the cellular response to shear stress.

METHODS

Device Fabrication and Assembly:

The parallel plate bioreactor provides a fluid flow path of adjustable height across the apical side of the cells and a static reservoir on the basal side (Fig 3.1)[15]. Each device is composed of three layers, a base with a 5 mL apical reservoir, a middle plate to hold the Snapwell insert (Costar,

Corning) with 1.12 cm^2 cell area and a top plate containing a 1-2 mL basal reservoir. The plates were machined initially from polycarbonate and later from polysulfone to allow for sterilization by autoclaving. Silicone gaskets (McMaster-Carr) of 500-1000 µm height were sandwiched between each set of plates to seal the fluid compartments and on the apical side to define the flow height. The bioreactor was compressed with screws. Depending on the iteration of the device, the reservoir and bubbletrap were either an external tube or a built-in column. The inlets and outlets were connected with Masterflex LS-14 silicone tubing with 1.6 mm inner diameter (Cole Parmer). Fluid flow and thus apical shear stress were set and controlled by a peristaltic pump (Cole Parmer).

Cell Culture and Flow:

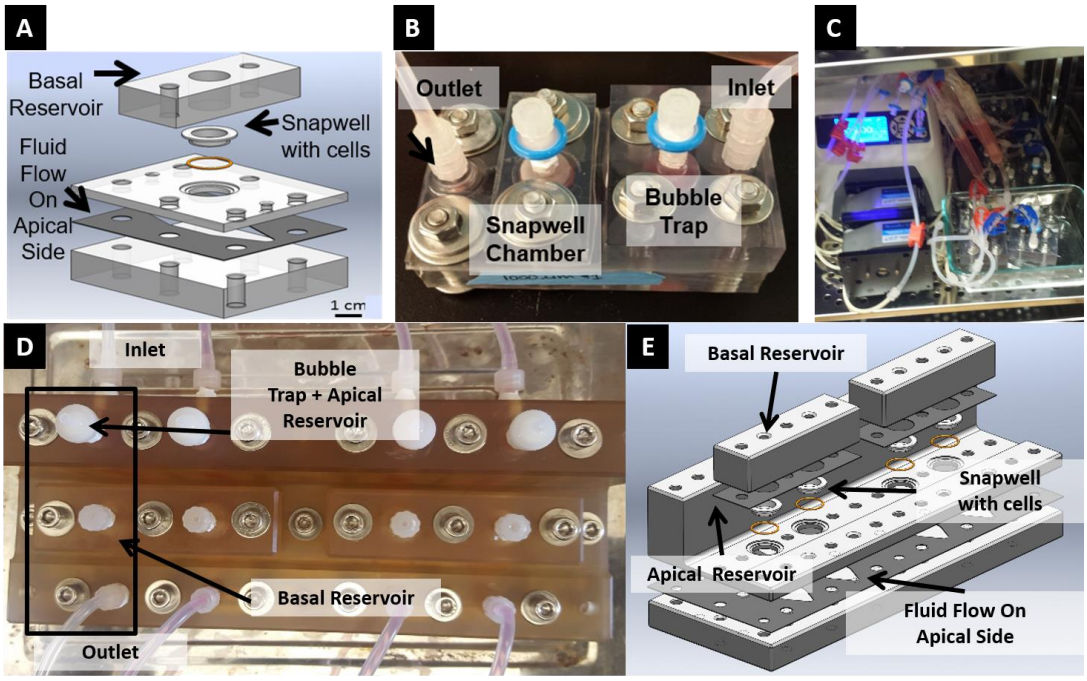

Figure 3.1. Bioreactor Design. A) First iteration of device with open basal reservoir and no built in bubbletrap. B) Second generation of device with built in bubble trap. C) Entire set up including pump and bubbletrap. D) Top down view of the four-channel device. Boxed area indicates one channel with inlet, 5 mL apical reservoir with bubble trap, outlet and a 1-2 mL basal reservoir; *E*) Exploded schematic of the four-channel device set up with 1.12 cm^2 cell area, gasket (500-1000 µm) defined apical fluid flow path and basal reservoir shown.

MDCK cells transfected with either an empty vector or a pair of uptake and efflux transporters (hOCT2/hMATE1)[16] were kindly provided by Dr. Martin Fromm (Stuttgart, Germany) and cultured in MEM with Earle's BSS (UCSF Cell Culture Facility) with 10% FBS (Gibco, Thermo Fisher Scientific) and 1% penicillin-streptomycin (UCSF Cell Culture Facility); 500 μg/mL hygromycin (UCSF Cell Culture Facility) and 100 mg/mL geneticin (Sigma-Aldrich) were added to the media for the double transfected cells. For flow experiments, hOCT2/hMATE1 MDCK cells were plated on Snapwell inserts at a density of 300,000 cells/well, grown under static conditions until confluence and then placed in the bioreactor. Media flow was increased over 7 days from 0.1 mL/min to 1-6 ml/min until desired shear stress was achieved. Cells were grown under static conditions for the same amount of time as controls.

Immunofluorescence:

Cells were fixed with 4% paraformaldehyde (Pierce, Thermo Fisher Scientific), permeabilized with 0.1% Triton-X in PBS (Sigma-Aldrich) and blocked with BSA (Sigma-Aldrich). They were subsequently incubated with 1:50 dilution of Alexa 488-labeled zonula occludens 1 (ZO-1) mouse monoclonal antibody (Life Technologies) or a primary acetylated α-tubulin mouse monoclonal antibody (Life Technologies) for 60 minutes. Cells treated with the α-tubulin antibody were then incubated with a secondary 561 anti-mouse goat antibody (Life Technologies) and phalloidin (Thermo Fisher Scientific) for F-actin staining. Imaging was performed using a Nikon spectral confocal microscope with a 40x oil objective.

Barrier Performance:

FITC-labeled inulin (Sigma-Aldrich) was added to the apical media and allowed to flow through the devices. Samples were collected from the basal reservoir every 24 hours and analyzed for inulin content using a Genios Pro fluorescence plate reader (Tecan). Barrier function of the cell monolayer was calculated from inulin levels measured in the apical and basal compartments as

described by Equation 3.1. Here, C_{apical} is the concentration of inulin in the apical compartment and C_{basal} is the concentration in the basal compartment. Inulin leak was calculated as concentration in the basal (donor) compartment divided by the cell area.

$$\textbf{Barrier Performance} = \frac{\mathbf{C_{apical} - C_{basal}}}{\mathbf{C_{apical}}}$$

Equation 3.1

ASP+ Transport:

Cells were induced with 10 mM Na-butyrate (Sigma-Aldrich) 24 hours prior to a transport experiment, as described previously.[16] When appropriate, cells were incubated with an inhibitor (500 μM imipramine or 1 mM cimetidine (Sigma-Aldrich)) on the basal side for 30 minutes, followed by addition of 25 μM 4-(4-dimethylamino)styryl-N-methylpyridinium (ASP+) (Life Technologies) and incubation for 1 hour. Samples were collected from the apical and basal media and cells were lysed for measurement of ASP+ accumulation and transport. Transport experiments were simultaneously performed on cells under shear stress and cultured under static conditions. ASP+ content was quantified on a Genios Pro fluorescence plate reader (Tecan) at an excitation wavelength of 485 nm and an emission wavelength of 590 nm.

Protein Quantification:

Cells were lysed with 1% SDS-10 M NaOH lysis buffer while shaking overnight. Protein content was measured using a standard Pierce BCA protein assay kit (Thermo Fisher). Where appropriate, protein content was normalized by cell growth area.

RNA Expression:

RNA was extracted from cells using an RNeasy RNA Extraction Kit (Qiagen) and cDNA was generated using an iScript kit (Bio-Rad). cDNA was used for detection of human and dog OCT2 and MATE1 and dog P-gp by qRT-PCR using a Taqman assay and probes (Applied Biosystems)

on the Fast Realtime PCR instrument (Applied Biosystems). RS-18 was used as a housekeeping control. The effect of shear stress on transporter expression was analyzed using the ΔΔCt method using transporter levels expressed relative to RS-18.[17,18] P-gp was used as a measure of global effects of shear stress on transporter expression.

Deciliation:

Cells were grown to confluency and then incubated in the absence or presence of 10 mM ammonium sulfate (Fluka AG) for 24 hours prior to measurement of ASP+ transport. The presence of cilia was determined by imaging of α-tubulin as described above.

Statistics:

All experiments were performed in triplicate with a minimum of two technical replicates within each experiment. Data are expressed as mean ± standard deviation and graphed as box and whisker plots. Statistical analyses were performed by unpaired one way or two way ANOVA and a p value of <0.05 was considered significant. Data were analyzed using Prism Version 6.0 (Graphpad).

RESULTS

Device optimization:

Several generations of the device were tested before identifying a configuration that was successful. The initial circuit was composed of a polycarbonate device, a separate reservoir and a bubble trap (Fig. 3.1A-C). The reservoir, a 50 mL falcon tube sealed with a PDMS block with holes punched through for tubing, was vulnerable to microbial contamination and leaks. The bubble trap, initially made with laser acrylic pieces glued together with acrylic glue, also encountered issues of leakiness. The bubble trap was eventually switched to a commercial trap used for patient dialysis (Nipro dialysis tubing A209Y), which significantly improved issues with leakiness. In the second generation device, a small built in bubble trap was incorporated along the flow path right before the Snapwell but this trap was too small to be effective and would regularly

fill up with the perfusate (Fig. 3.1B-C). Additionally, with the polycarbonate versions of the device, sterilization was a major issue. All the silicone and metal parts were initially sterilized by autoclaving and the polycarbonate and acrylic by either autoclaving or immersing in a 10% bleach solution followed by a 70% ethanol solution. Both methods caused the plastics to crack within a few uses and increased their vulnerability to cracking during compression.

In the final generation of the device, the material was switched to polysulfone, which was autoclavable. In addition, a larger reservoir-bubble trap combination was incorporated into the device itself, which eliminated the complexity and greater infection risk of multiple disjointed parts (Fig. 3.1D, E). Multiple channels were also added into the device so assembly of replicates or different conditions was much faster. Lastly, for sterilization, all parts were autoclaved and sterile gloves were used to completely eliminate microbial contamination.

Cell Morphology and Barrier Function:

The conditions for ramping up the fluid flow rate were optimized to maintain cell integrity. It took a lot of trial and error to determine the rate at which fluid flow could be ramped up without destroying the cell monolayer. Starting at the desired end flow rate caused cells to slough off the Snapwell as measured by inulin leakiness. However, slow rates of flow increase unreasonably prolonged the experiment and increased risk of infection, tubing degradation and cell death. Attempts at controlling flow rate increases using software were limited by the requirement to reprogram every 24 hours. A final protocol of 7 days of daily ramping to achieve 0.2- 2 dynes/cm^2 of shear was used across all studies reported below.

Immunofluorescence analysis of cells placed under flow revealed uniform monolayer formation with the tight junction protein, Zonula-occludens-1 (ZO-1), localized to the tight junctions between cells under both static and flow conditions (Fig 3.2A-C). Quantification of total protein content demonstrated up to a 1.6-fold increase in cell content in response to shear stress

(Fig 3.2D). Next, permeability of inulin, a marker of proximal tubule leakiness, across the monolayer was measured. Devices retained a barrier performance rate of 97.9 ± 1.4% over 7 days of culture under up to 2 dynes/cm^2 of shear stress (Fig 3.2E). This resulted in a final inulin leak rate on day 6 of 0.13-0.69 µg/cm^2/day.

Effect of Shear Stress on Organic Cation Transport:

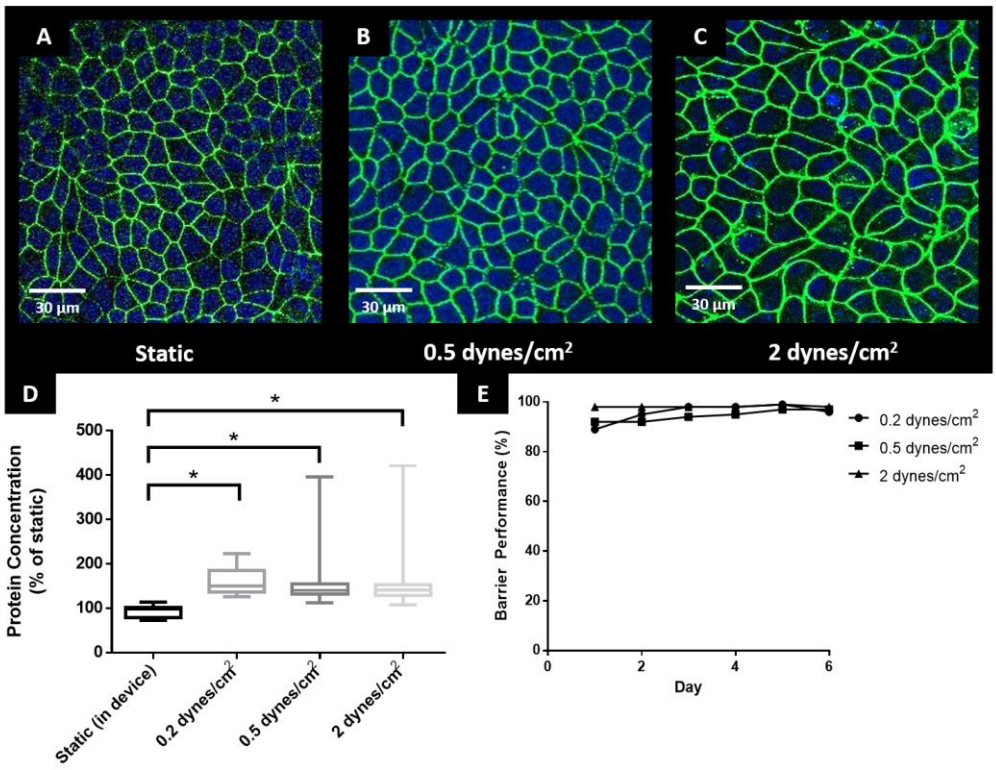

Figure 3.2. Effects of Shear Stress on MDCK Cell Monolayer Formation. A-C) Nucleus (DAPI) and tight junction (ZO-1) stains of cells under **A)** no, **B)** 0.5 dynes/cm^2 and **C)** 2.0 dynes/cm^2 levels of shear stress for 72 hours; Blue = DAPI for nucleus and Green = ZO-1; **D)** Protein content of cells under varying flow conditions for 72 hours. Experiments were performed in triplicate and data are shown as box (median with 25th to 75th percentile) and whiskers (min and max); * indicates $p<0.05$; **E)** Barrier performance of cells under flow as measured by inulin permeability across the monolayer every 24 hours for 6 days. Data presented are from a single representative device at each shear stress level.

There were multiple challenges in measuring transport in the devices. Completely replacing the media in the devices with HBSS buffer before performing the assay was time consuming and difficult. Media replacement also often washed the cells from the Snapwell insert. Additionally, media replacement required fluid flow to be stopped and restarted multiple times, which damaged the cell monolayer. In the final iteration of the assay, transport assays were conducted in media with no disruptions in fluid flow; these conditions significantly improved the viability of the cell monolayer.

Transport of ASP+, an auto-fluorescent substrate of OCT2 and MATE1, was measured for one hour at increasing levels of shear stress (0.2-2 dynes/cm^2). MDCK cells exogenously expressing hOCT2 and hMATE1 showed a 4.2-fold increase in ASP+ transport in response to shear stress of 2 dynes/cm^2 as reflected in measures of both cellular accumulation (Fig 3.3A) and transcellular transport (Fig 3.3B). The effects of shear stress on ASP+ accumulation and transcellular transport were similar under shear stress of 0.5 dynes/cm^2 and 2 dynes/cm^2. To determine if active ASP+ transport was increased, transport of ASP+ by cells exposed to 0.2 dynes/cm^2 of shear stress was measured with or without pretreatment with 500 μM imipramine, an OCT2 and MATE1 specific inhibitor. ASP+ transport was inhibited by imipramine 60.3 ± 15.8% under shear stress conditions compared to 47.6 ± 19.7% under static conditions (Fig 3.3C).

To further understand this observation, the effect of shear stress on the expression of human OCT2 (transfected), canine OCT2 (endogenous) and canine P-gp (endogenous) was measured in cells exposed to varying levels of shear for 72 hours (Fig 3.4). In comparison to cells cultured under static conditions, MDCK cells exposed to shear showed increased expression of transfected

human and endogenous OCT2 (up to 3.7- and 2.0-fold, respectively), with no significant effect on the expression of transfected MATE1 or endogenous P-gp.

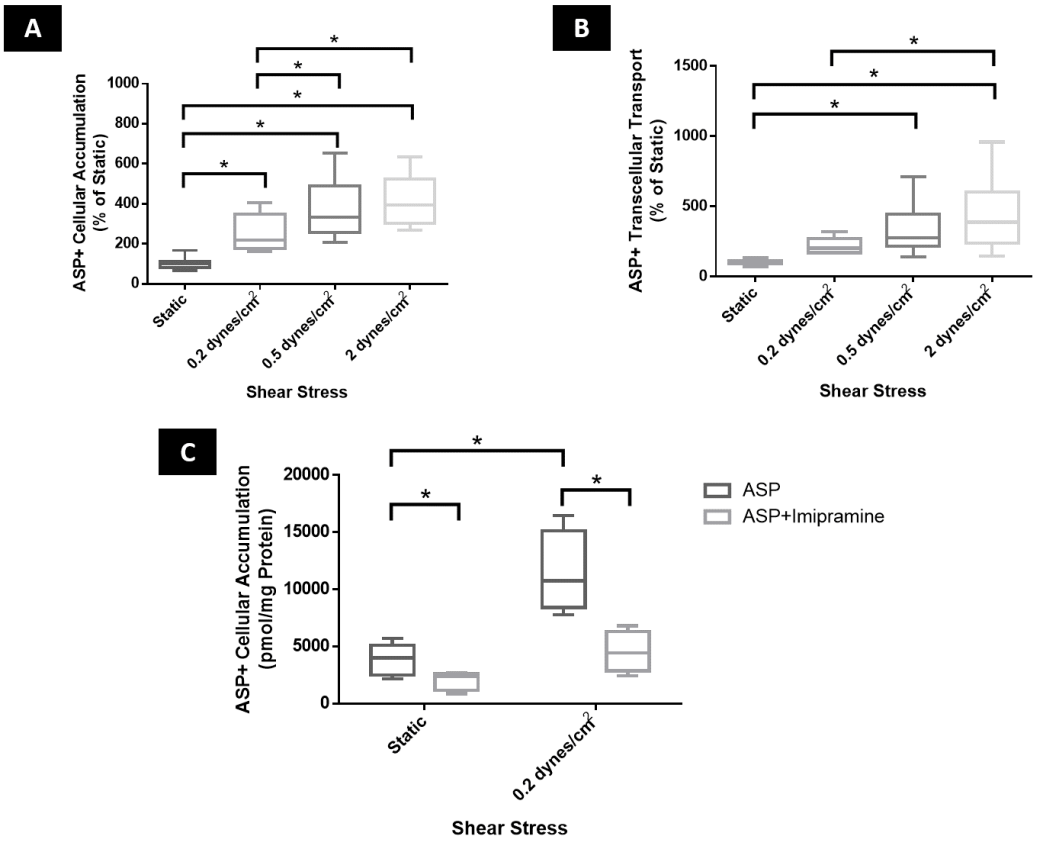

Figure 3.3. ASP+ Transport in hOCT2/hMATE1 Double Transfected MDCK Cells Exposed to Shear Stress. **A)** Accumulation and **B)** transcellular transport of ASP+ by a monolayer of cells exposed to 0-2 dynes/cm^2 of shear stress for 72 hours followed by basolateral addition of ASP+ for 1 hour. Data are expressed as percent relative to accumulation or transport by cells cultured under static conditions **C)** ASP+ accumulation into cells exposed to 0-0.2 dynes/cm^2 of shear stress for 72 hours followed by basolateral addition of ASP+ for 1 hour with or without exposure to an inhibitor. Experiments were performed in triplicate and data are shown as box (median with 25th to 75th percentile) and whiskers (min and max); * indicates $p<0.05$.

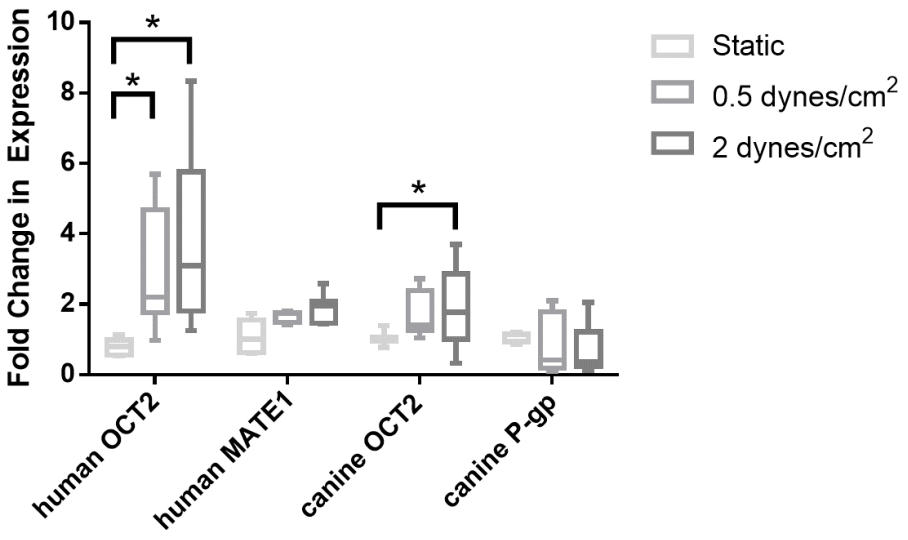

Figure 3.4. Effect of Shear Stress on RNA Expression of Transporters. Human (transfected) and endogenous canine OCT2 (organic cation transporter), transfected human MATE1 and canine P-gp mRNA levels were measured in hOCT2/hMATE1 double transfected MDCK cells. Cells were exposed to 0-2 dynes/cm^2 for 72 hours before RNA expression was measured. Experiments were performed in triplicate and data are shown as box (median with 25th to 75th percentile) and whiskers (min and max); * indicates $p<0.05$.

Role of Cilia in Response to Shear:

Imaging showed that double transfected MDCK cells express cilia under both static and flow conditions (Fig 3.5A,E). Exposure of the cells to 30 mM ammonium sulfate for 24 hours caused a complete loss of cilia (Fig 3.5C,G). Importantly, deciliation had no effect on cell membrane tight junctions, as measured by imaging of F-actin, which anchors with tight junction proteins (Figures 3.5D and 3.5H)[19]. Deciliation had no effect on transport by cells cultured under

static conditions but completely eliminated the effects of shear stress on ASP+ transport in cells exposed to 0.5 dynes/cm^2 of shear (Fig 3.6).

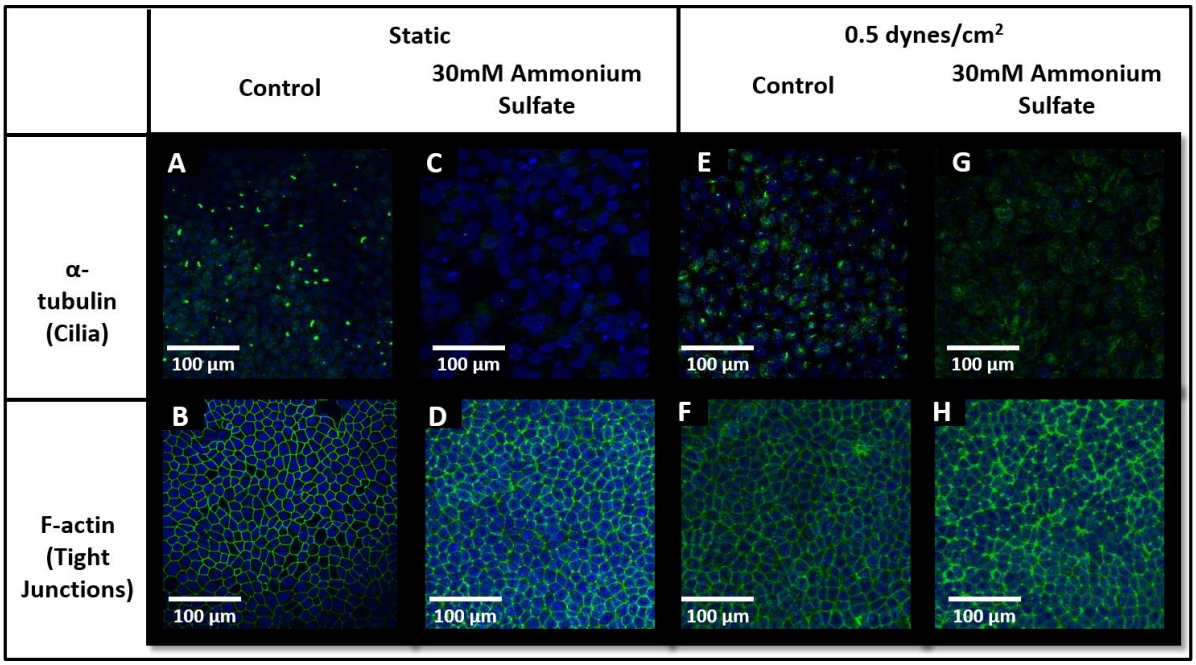

Figure 3.5. Effect of Deciliation on Morphology of DT-MDCK Cells. Staining of nuclei (DAPI) and cilia (α-tubulin) or tight junctions (F-actin) in cells exposed to 0-0.5 dynes/cm^2 for 72 hours and where labeled, 30 mM ammonium sulfate for 24 hours. **A,C,E, and G)** Blue = DAPI, Green = α-tubulin; **B,D,F, and H)** Blue = DAPI, Green = F-actin.

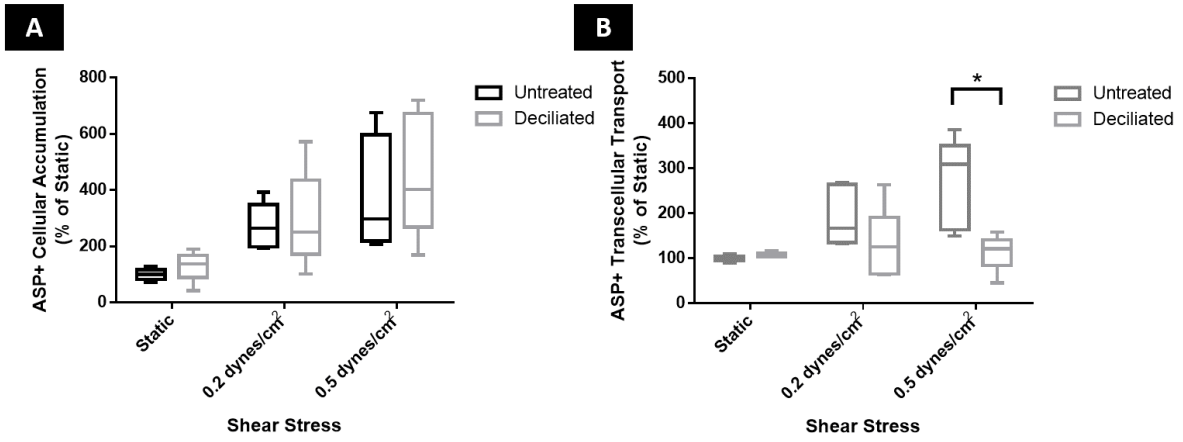

Figure 3.6. **Effect of Deciliation on ASP+ Accumulation and Transport by hOCT2/hMATE1 Double Transfected MDCK Cells. A)** Accumulation and **B)** transcellular transport of ASP+ by a monolayer of cells exposed to 0-0.5 dynes/cm^2 of shear stress for 72 hours and 30 mM ammonium sulfate for 24 hours as indicated (deciliated), followed by basolateral addition of ASP+ for 1 hour. Data are expressed in percent relative to accumulation or transport by cells cultured under static conditions. Experiments were performed in triplicate and data are shown as box (median with 25th to 75th percentile) and whiskers (min and max); * indicates $p<0.05$.

DISCUSSION

The kidney plays a central role in the elimination of drugs in the human body. It is important to accurately mimic the complexity of renal physiology for *in vitro* drug testing, particularly with respect to fluid flow. Shear stress from fluid flow has been shown to affect cell morphology and ion transporters *in vitro*, but little is known about the effects on drug transporters[9,11,20]. Additionally, most previous studies have only measured the effect of short-term shear (1-6 hrs), which is unlikely to fully separate shear stress effects from other cellular stress

responses[9,13,14]. Understanding how drug transporters respond to varying levels of sustained shear exposure can help improve *in vitro* predictions of *in vivo* drug handling by the kidney. Accurately designed *in vitro* models of the kidney are expected to standardize preclinical testing and reduce drug failure rates.

In this work, a parallel plate bioreactor was used to elucidate the effects of sustained shear stress on organic cation transport by OCT2 and MATE1 in renal cells. Several cell lines were considered for these studies, including human cell lines such as HPCTs from the Hopfer laboratory[21] and RPTECs (ATCC CRL-4031)[22], but the double transfected hOCT2/hMATE1 MDCK cells were considered the most appropriate choice for this study for several reasons. First, the MDCK cells consistently demonstrate robust attachment to the transwell inserts, which allows them to withstand the initial stress of fluid flow. Second, these cells form tight monolayers, which prevents leakiness and is essential for study of transcellular transport of marker substrates. Finally, exogenous expression of transporters allows for improved detection of active transport and the effect of perturbations.

Cells placed under flow formed a confluent monolayer and displayed clear tight junctions. They also retained high barrier function as measured by inulin permeability. Inulin, a 5000 Da polymer, is a well-known marker of glomerular filtration rate and is considered a marker of the leakiness of the proximal tubule *in vivo*[23]. The ability of epithelial cells to form a monolayer that prevents fluid and protein leak is critical for proper function of the tubule. Here, the leak through the MDCK cells was minimal at less than 1 $\mu g/cm^2$/day and significantly lower than that through human cells, which our collaborators showed to be approximately 10-20 $\mu g/cm^2$/day, supporting the conclusion that they formed a robust monolayer[15].

Transport of ASP+ was significantly increased in hOCT2/hMATE1 MDCK cells exposed to varying levels of shear stress for 72 hours when compared to static controls. ASP+ is taken up

on the basolateral membrane by OCT2 (*SLC22A2*) and effluxed on the apical membrane by MATE1 (*SLC47A1*), two organic cation transporters that work in concert to facilitate the renal secretion of commonly used drugs such as metformin and cisplatin[24,25]. Similar effects of shear stress have been reported for proximal tubule ion transporters. Increases in albumin uptake, ion reabsorption and megalin and cubulin expression and function have been reported in response to increased shear stress[9,10,12,20]. Exposure of renal proximal tubule cells to shear stress is also associated with reduced apoptosis and faster recovery from acute cisplatin toxicity and enhanced inhibition of organic anion transport[13]. Since shear stress from fluid flow is constantly present in the proximal tubule, these findings collectively support the use of more physiological *in vitro* model systems to predict renal drug disposition and toxicity. It is interesting to note that there is no significant difference in transporter function between 0.5 dynes/cm^2 and 2.0 dynes/cm^2 of shear stress. Prior studies by Essig et al. had found that a minimum level of shear of 0.17 dynes/cm^2 is required to elicit an alteration in cell morphology and other studies have measured effects on physiology at up to 1 dyne/cm^2. [7,9] However, this is the first study to explore the effects of a range of higher and sustained shear stress levels on the functionality of renal drug transporters. While further investigation is required, the results shed light on the required conditions for physiologically relevant models and the effects of increased shear stress levels due to disease on renal drug handling.

Surprisingly, the RNA expression of transfected human and endogenous OCT2 was increased in cells exposed to all levels of shear stress compared to static controls. This upregulation of transporter expression was specific, with no measurable effect on endogenous P-gp expression or transfected MATE1. The upregulation of transporter expression gives insight into the mechanism behind the increased transport and may be a result of enhanced mRNA stability or increased mRNA transcription. It is interesting to note that both the transfected and endogenous

transporters were upregulated, which is unexpected since transfected OCT2 was expressed under a CMV promoter and should not be subject to endogenous gene expression regulatory mechanisms. While surprising, this observation is not unprecedented. A similar effect on OAT1 transfected proximal tubule cells exposed to perfusion has been reported but the mechanism for the increased expression remains unclear[11]. In another study, Nrf2 signaling was found to play a role in increasing endogenous MATE2-K expression in response to shear stress[26]. Further study into this is warranted and will be the subject of future investigations.

Cilia are known to have mechanosensory roles in the proximal tubule[27]. Therefore, to determine whether they play a critical role in the increased OCT2 and MATE1-mediated transport reported here, the effect of cilia removal on transporter function was measured. The complete blockade of shear-dependent increases in ASP+ transport by removal of cilia is similar to the effect found in studies by Raghavan et al. who demonstrated a cilia dependent upregulation in endocytosis in response to fluid flow[10]. It is important to note that ammonium sulfate may have other uncharacterized effects on global cell behavior and functionality that could indirectly impact solute transport. While this method of deciliation has limitations, with appropriate controls it still allows an exploration of the role of ciliary sensing on transporter-mediated movement of organic solutes. The mechanism of signaling between the mechanosensory proteins in the cilia and the transporter is currently unknown. One hypothesis is that function of the organic cation transporters is altered due to altered ion transport. Removal of cilia is known to alter solute motility in the proximal tubule cells, specifically by reducing Na^+/K^+-ATPase membrane localization and modifying paracellular transport[12]. It is possible that this results in alterations in MATE1 function, an antiporter dependent on the H^+ gradient. Another hypothesis is that sensing of shear stress affects expression of organic cation transporters. It has been found that shear stress modulates MATE2-K function through Nrf2 signaling[26]. Other organic cation transporters may respond

similarly to shear stress, which would be eliminated when the mechanosensory cilia are removed. Much of this is unknown and warrants further study. Interestingly, cilia removal did not have a significant effect on ASP+ transport by cells exposed to 0.2 dynes/cm^2 of shear stress, but a robust effect was observed when shear stress was increased to 0.5 dynes/cm^2. This suggests a threshold shear stress level is sensed by the cilia, which in turn signal changes in transporter expression and function before measurable effects on transport are expected. Overall, the dependence of organic cation transport on ciliary sensing of shear stress provides insight into the mechanosensory signaling pathway involved and the minimum level of stress that might be required to trigger a robust response to shear.

CONCLUSION

In summary, these data demonstrate that shear stress from fluid flow has a significant effect on organic cation transporter function and expression in MDCK cells. Furthermore, upregulation of organic cation transport was dependent on the presence of cilia. We propose that apical shear stress is an important component of any *in vitro* modeling of renal tubular cells and is likely to be an important component of modeling renal secretory clearance and nephrotoxicity of drugs. The specific mechanism by which mechanical stress signals increases transporter activity and expression is still unclear and will require further study.

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CHAPTER 4:

DESIGN OF A SCALED MODEL OF THE GLOMERULUS AND PROXIMAL TUBULE

INTRODUCTION

Disposition of novel drugs in the body is currently determined mainly through pre-clinical animal studies. However, animals are poor mimics of human drug disposition for several reasons. First, there are size differences between species that contributes to differences in clearance. Furthermore, there are substantial variations in expression and tissue distribution of transporters and metabolic enzymes that significantly limit the prediction of human drug transport and clearance rates from animal data[1]. One proposal for overcoming these issues is to bypass animal testing, and instead, develop *in vitro* human-on-a-chip models that accurately represent the human body.

In theory, human-on-a-chip models contain compartments that mimic each organ and connect them together in a physiologically relevant manner through a fluidic vascular system. Development of individual organ on a chip systems has progressed significantly and some initial work has been done to combine a limited number of compartments, but the interconnection of organs come with a unique set of challenges that have yet to be addressed, the most important of which is inter-organ scaling[2–4]. Within the human body, the size of every organ is proportional to the overall size of the human. However, in the past, when researchers have attempted to develop individual organs on a chip, there has been little attention paid to consistency in sizing between

groups. Recent inter-group collaborations have therefore focused on building organs that are parametrically matched such that the different systems can eventually be connected[4,5].

There are two approached that may be applied to scale organs to maintain the proportions of the human body. The first (and simpler) approach is allometric scaling, wherein organ sizes are scaled purely based on mass or surface area[6]. This scaling traditionally has been applied to estimation of dosing levels in both *in vitro* and *in vivo* models but is limited because organ functionality does not always scale with size. As a result, the second (and better) approach to scaling organs may be to consider instead, replication of some portion of organ function, wherein a representative parameter is selected and the organ model is scaled according to that parameter. For example, the heart in a milli-scaled organ on a chip would pump 1/1000th of the volume of a full-scale human. This method is advantageous because it preserves the ratio of key functional parameters between organs while providing a straight-forward, quantifiable metric for scaling[4].

In this chapter, functional scaling is applied to develop a 1:1000 scaled model of the kidney, specifically the glomerulus and proximal tubule. While it is important to model all parts of the kidney, the glomerulus and proximal tubule encounter and transport the highest concentrations of drug and are therefore the most important components for a first prototype[7]. In the nephrons, blood enters through the glomerulus, which is composed of podocytes, endothelial cells and a basement membrane. Together, these provide a semi-permeable, size and charge selective barrier with slit-shaped pores that filter out proteins while allowing free flow of water and small solutes. The remaining filtrate then travels to the proximal tubule where reabsorption and secretion occur. The tubule is made of a monolayer of epithelial cells that surround a lumen through which filtrate flows. The basolateral side of the monolayer is adjacent to capillaries and xenobiotics continuously

move between blood and filtrate. Ideally, a kidney-on-a-chip model of the two segments would incorporate all of these above-mentioned aspects.

No scaled models of the glomerulus exist but since the glomerulus has a straightforward function as a filter, much research has gone into designing membranes that can serve as acceptable mimics. Recently, our lab has designed silicon nanopore membranes (SNMs) that are ultrathin membranes with micromachined pores with uniform size distribution. The slit shape and size consistency of the pores are much better mimics of the glomerular membrane than traditional polymer membranes which comprise of circular pores of varying sizes. Furthermore, filtration experiments have demonstrated that the SNMs have superior hydraulic permeability, molecular selectivity and blood compatibility when coated with polyethylene glycol[8]. These membranes will be used as part of the glomerular compartment. While no scaled models of the proximal tubule exist, prior research on general proximal tubule models was discussed in Chapter 1. The model presented here draws from existing research and improves upon it to generate a scaled version of the proximal tubule. Initial prototyping efforts for the proximal tubule are presented but development of the model was discontinued because the organ on a chip field has shifted away from scaled models towards high throughput models. Reasons for this shift are discussed later in this chapter.

DESIGN

The proposed device will be composed of two segments, one for the glomerulus and one for the proximal tubule (Fig 4.1). The segments will be connected to a peristaltic pump and a blood reservoir with tubing and will have ports for sampling. The total fluidic volume of the model will be 5 mL, consistent with the 1:1000 scaling of the approximately 5 L human body blood volume.

Glomerular Segment:

Design: The glomerulus segment will consist of SNMs lined on one side with blood flow channels and on the other side by a filtrate channel, where filtrate can be collected and directed to the proximal tubule segment. SNMs will be fabricated using previously published methods[8]. The blood flow and filtrate channels will be fabricated out of polydimethylsiloxane (PDMS) using established soft lithography techniques[9]. The channels will then be attached to the SNMs through oxygen plasma bonding.

Design Parameters: The main function of the glomerulus is filtration. Glomeruli receive approximately 550 ml/min (of 5 L total volume) of blood at 30 mmHg pressure and produce filtrate at the rate of 45-62.5 ml/min[8,10]. Therefore, a milli-model will need to have the appropriate membrane area to produce at least 45 μL/min of filtrate. Since silicon nanopore membranes can transport 90 mL/min/m^2 at a driving pressure of 30 mmHg (based on previous work from our lab), the required membrane area for the glomerular segment is approximately 20 cm^2 including support structures. The blood channel will be divided into four smaller channels that are 1 cm by 5 cm (width and length) in dimensions to allow for adequate membrane support. In order to ensure the glomerulus receives 10% of total systemic volume (as in humans) while minimizing the pressure drop through the channel, the channel heights in the segment will be approximately 50 μm.

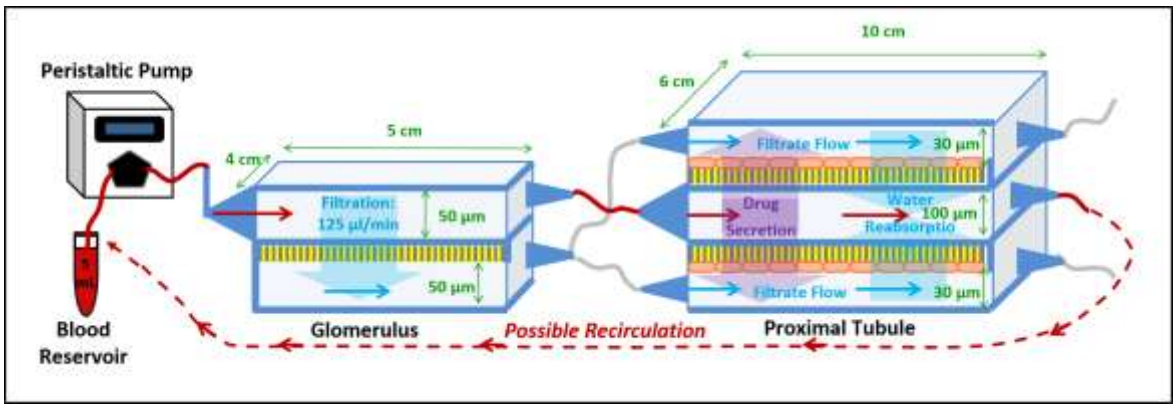

Figure 4.1 Design of a 1:1000 Scaled Glomerulus and Proximal Tubule. The glomerulus segment will be composed of one filtrate and one blood flow channel made of polydimethylsiloxane between which silicon nanopore membranes will be sandwiched. The proximal tubule segment will be composed of two filtrate and one blood flow channel made of polydimethylsiloxane. Between each layer, a polycarbonate membrane will be placed and cells will be seeded on the side facing the filtrate channels. The segments will be interconnected as depicted and also connected to a fluidic reservoir and a peristaltic pump.

Proximal Tubule Segment:

Design: In the proximal tubule segment, a 10 µm thick polycarbonate membrane with 0.4 µm pores lined with cells will be sandwiched between PDMS channels to allow transport between blood and filtrate. The sandwich will be repeated such that there is one inner blood flow channel and two outer filtrate channels in order to maximize efficient use of space and maintain fluid volume within the scaling constraints. The inner channel will be created by using PDMS stamping to apply a thin layer of patterned PDMS onto the polycarbonate membrane[11]. The coating and bonding techniques will need to be optimized to attain the desired channel height. Finally, oxygen plasma, epoxy glue or compression will be used to connect the various layers together via PDMS-PDMS bonds. Cells will be seeded on the side of the membranes facing the filtrate channel by injection into the channel

and incubation for a few hours. Since cell do not adhere well to PDMS, cells not attached to the membrane can then be washed off.

Design Parameters: The proximal tubule has multiple vital roles in the body and therefore, there are several functional metrics by which to scale a model. Here, scaling is based on water reabsorption but transport of solutes or drugs can also be used depending on the functional limitations of the cell line being considered. The proximal tubule reabsorbs about 60% of all the water that flows across it, meaning a minimum of 27-37.5 μL/min in a milli-model[13]. Given that *in vitro*, proximal tubule cells transport water at approximately 5 ml/min/m^2 (based on previous work from our lab), this segment will require a cell surface area of 60 cm^2. Additionally, proximal tubule cells face a shear stress of around 1-2 dynes/cm^2, which is required for proper cell function[12]. In order to account for reabsorption as well as shear stress requirements, the filtrate channels will be divided into 3 segments of approximately 2 cm by 10 cm by 30 μm (width, length and height) in dimensions and the blood channel will be similarly divided into 3 segments with a height of 100 μm.

EXPERIMENTAL METHODS

Device Fabrication:

To create the flow channels for the prototype, standard soft lithography techniques were used. In short, a photolithography mask was designed using AutoCAD (AutoDesk). The mask was used to create a master mold through UV polymerization of photoresist (SU-8 100, Microchem, Newton, MA) spun on the surface of a silicon wafer. Polydimethylsiloxane (PDMS, Sylgard, Dow Corning) was mixed at a 1:10 ratio of curing agent to pre-polymer and cast onto the mold for 12 hours at 60°C. This method of fabrication will be used to create most of the channels in the final scaled device as well.

To stamp the inner channels in the proximal tubule model, a similar molding process will be used to create PDMS stamps. The stamp will then be coated with an uncoated PDMS layer of the same composition and allowed to cure for 12 hours at 60°C. The stamp with the thin layer and the polycarbonate membrane (Corning) will then be oxygen plasma treated and attached. After bonding, the larger stamp layer will be peeled away. The exposed side of the thin layer can be plasma treated again and attached to another layer of polycarbonate membrane.

The inlets and outlets will be punched out of the PDMS with a standard hole puncher and tubing will be connected with luer lock connectors.

Bonding Methods:

Oxygen Plasma: Cured PDMS channel layers and polycarbonate membranes were exposed to oxygen plasma for 30 seconds at 100 watts and then placed together and allowed to bond for one hour.

Compression: A separate module was created to allow for compression based sealing. This module consisted of two acrylic pieces cut to size with four holes for screws. Parallel holes were cut into the PDMS channel layers. The layers were aligned and compressed by tightening of screws.

PDMS Glue: Cured PDMS channel layers were hand coated with a brush with uncured PDMS (1:10 curing agent:prepolymer ratio) and immediately attached to a polycarbonate membrane placed in an external holder (embroidery hoop) to minimize wrinkling. The sandwiched device was cured for 12 hours at 60°.

Epoxy Glue: Cured PDMS channel layers were hand coated with a brush with Loctite epoxy and immediately attached to a polycarbonate membrane placed in an external holder (embroidery hoop) to minimize wrinkling. The sandwiched device was then cured for 12 hours at 60°C.

Testing Bonding Methods: Once the bonding has been completed, a syringe filled with colored water was attached to the inlet via tubing and luer lock connectors. Bond strength was tested by flowing fluid through the channels at a rate of approximately 1 ml/min.

Cell Culture and Flow:

In the full device, cells suspended in media will be loaded into the assembled bioreactor via a syringe and incubated for four days until confluency is reached. Following this, flow through the bioreactor will be initiated and increased over time until the desired flow is reached.

RESULTS

Fabrication of Proximal Tubule Segment:

An initial prototype of the proximal tubule was made with a single filtrate channel and a single blood channel in order to maintain simplicity while optimizing fabrication and bonding methods. The schematic and photomask used to create the SU-8 based master mold are shown in Fig 4.2. The same master mold was used to generate both the blood and filtrate channels.

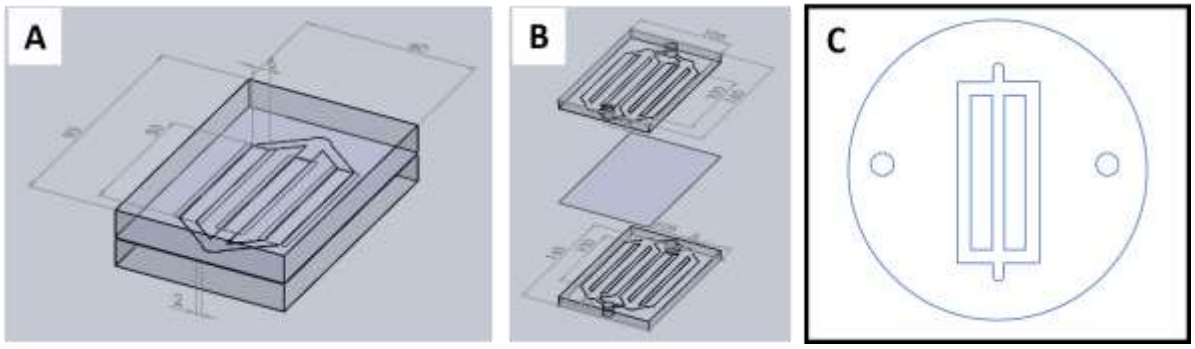

Figure 4.2 Schematic of Initial Proximal Tubule Prototype A) Condensed and B) exploded views of device consisting of one filtrate flow channel and one blood flow channel with a polycarbonate membrane sandwiched in between. C) Photomask design used to generate prototype.

Bonding of Proximal Tubule Layers:

Several bonding methods were attempted to properly seal the fluid flow channels onto the polycarbonate membrane, including oxygen plasma treatment, compression based sealing, PDMS glue and epoxy glue. Oxygen plasma treatment did not result in any attachment between the surfaces. Compression was attempted as depicted in Fig 4.3 and resulted in a tight seal but minimal fluid flow. Instead of flowing through the channels, the fluid broke through the polycarbonate membrane and into the opposite side of the device.

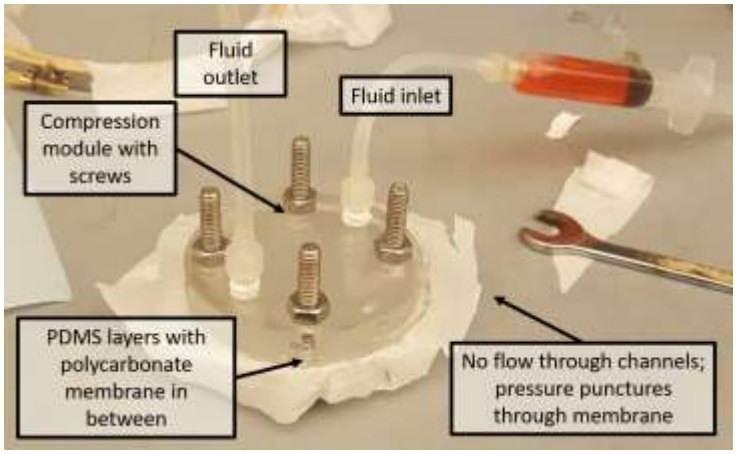

Figure 4.3 Bonding of Proximal Tubule Segment Through Compression. A compression module consisting of two acrylic layers held together by screws was used to compress the proximal tubule prototype composed of a polycarbonate membrane sandwiched between two PDMS fluid flow layers.

PDMS glue was easy to apply but leaked into the channel areas when applied to the device and attached to the polycarbonate membrane (Fig 4.4). Moreover, it provided a very weak bond that could be easily separated manually by hand and bonded devices were unable to maintain channel boundaries under fluid flow.

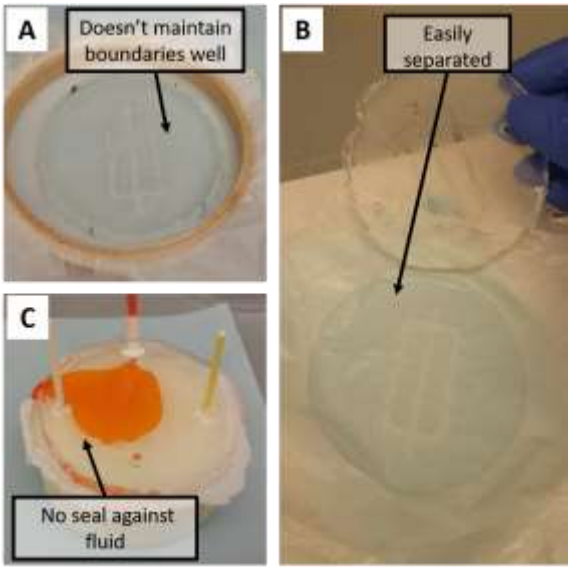

Figure 4.4 Flow Through Devices Bonded with PDMS Glue. A) The uncured PDMS glue leaks into channels when a polycarbonate membrane is placed on top. B) After 12 hours of curing, the layers are still easily separated by hand. C) The cured PDMS glue provides no barrier against fluid leakage.

Loctite epoxy was easy to apply and did not leak into channels upon contact with the polycarbonate membrane. Furthermore, the cured device remained bonded and was able to maintain channel boundaries under fluid flow. However, these boundaries weakened upon prolonged exposure (<12 hours) to fluid.

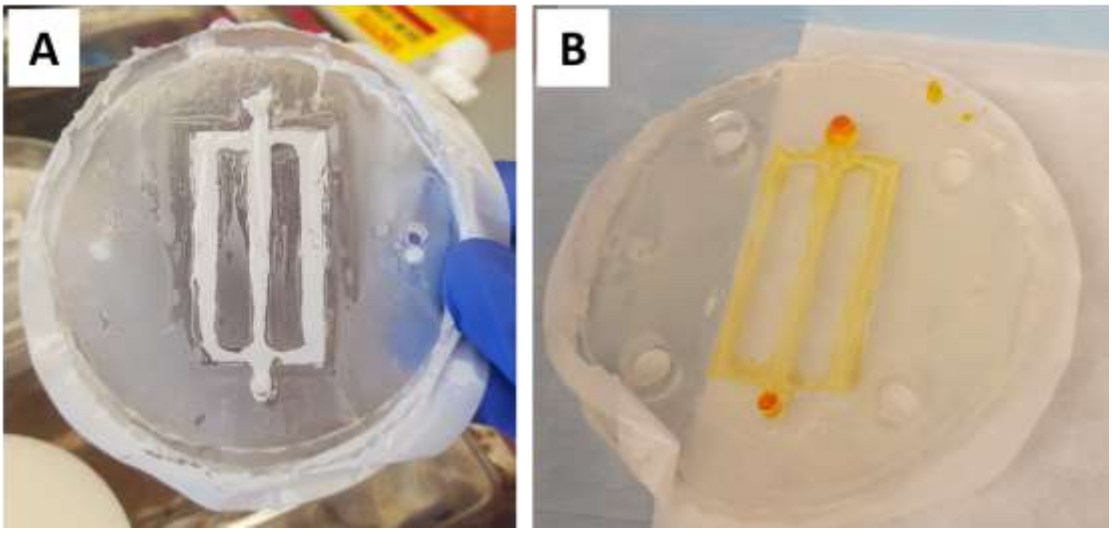

Figure 4.5 Flow Through Devices Bonded with Epoxy Glue. A) The uncured epoxy glue did not leak into channels upon contact with polycarbonate membrane B) After 12 hours of curing, the layers remained bonded and the glued areas provided a barrier to fluid leak.

DISCUSSION

In order to accurately replicate drug disposition *in vitro*, it is important to model the interaction between all organs of the body, particularly those relevant to drug handling such as the liver and kidney. Compartments in such a human-on-a-chip model need to be scaled such that the ratio of functionality between organs is preserved between the *in vivo* and *in vitro* settings. Here, design and parameters of a functionally scaled model of the components of the kidney relevant to drug handling, namely the glomerulus and proximal tubule, were proposed. In this design, each compartment is composed of a parallel plate bioreactor with fluid flow channels mimicking urine and filtrate flow in the kidney and filters providing either a sieving or growth surface. Physical parameters were determined based on filtration rate for the glomerular segment and water reabsorption for the proximal tubule segment, two critical functions of the kidney.

Initial prototyping efforts for the proximal tubule were undertaken including generation of PDMS based flow channels and bonding of channels to the polycarbonate membrane. Epoxy glue seemed to perform best in providing a seal between layers but the hand coating process resulted in non-uniform spreading of the epoxy that likely results in variable channel heights. To eliminate this issue, spin coating or stamping of epoxy onto the PDMS slabs should be considered. Additionally, the epoxy bonds weaken when exposed to constant flow over the course of a few hours. Therefore, although compression did not successfully enable fluid flow in the current system, it may be a better direction to pursue. The issue with the current design was that the flow path of least resistance was through the inlet opening and directly through the membrane to the inlet on the other side (Fig. 4.6A). The inlets were directly in line with each other, because of which the polycarbonate membrane sandwiched in between was completely unsupported by a PDMS backing. To overcome this, a new design with off-set inlets and outlets is proposed in Fig. 4.6B such that each opening is supported by solid PDMS. Compression bonding with this improved design should be pursued if this project is continued.

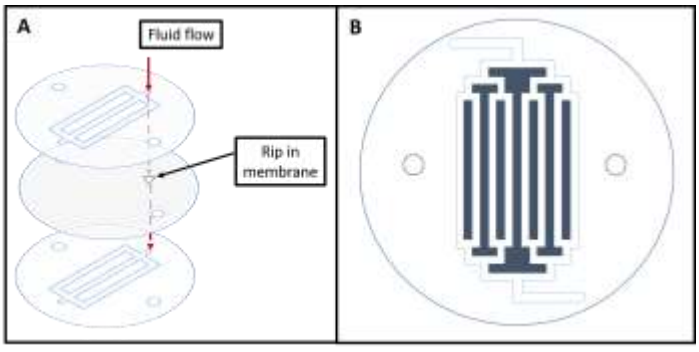

Figure 4.6 Schematic of Issues with Compression and Updated Proximal Tubule Design. A) When fluid flow was attempted after compression of device components, fluid ripped directly through the membrane and out the lower inlet. B) To overcome this issue, a new design is proposed with offset inlets and outlets supported by solid PDMS

In parallel, practical translatability of the scaling factor from theory into a usable physical model was considered. While the scaling of the glomerulus is straightforward given that function will be mechanically rather than biologically reproduced, it became evident over the course of the project that scaling of the proximal tubule would be problematic for several reasons. The cell lines explored in Chapter 2 did not show any evidence of water reabsorption when tested *in vitro.* In addition, few publications were found that could conclusively demonstrate water transport by cells cultured *in vitro*. Given this, other metrics of scaling were considered and two possibilities were transport of glucose, a substrate of reabsorption in the proximal tubule, and metformin, a well-known organic cation substrate secreted by OCT2 and MATE1 in the tubule. However, even if these measurements were feasible, the proximal tubule has multiple important functions and scaling by any one metric does not guarantee that other functions will also scale. As described in Chapters 1 and 2, cells available for *in vitro* use do not express all transporters at physiological levels and sometimes do not maintain the appropriate ratio of expression between transporters, making it unlikely that any one substrate can serve as a basis for scaling of a model. Therefore, it may be better to individualize scaled models to specific substrate types or active transport mechanisms such that separate models are used for each, eliminating the confounding factor of variable expression levels. This solution of course, significantly reduces the utility of a scaled model, particularly when considering incorporation into a larger human-on-a-chip system.

The project was suspended at this point. As mentioned above, scaling of organ components is problematic when considering complex systems with multiple vital functions. When the limitations of functionality of *in vitro* systems are also considered, building a scaled model becomes very difficult. If the goal is to build an accurate scaled model, it may be better to wait until more suitable cell sources are available or a better scaling metric is identified. The field

overall has also shifted focus towards high throughput models that can process a large number of drug candidates simultaneously. Many labs, academic-industry partnerships and recent start-ups such as Organovo, Mimetas and Emulate have all focused on creating high throughput kidney models for drug transport and nephrotoxicity screening. While scaled human-on-a-chip models are still the ideal substitute for animal testing, it is an ambitious goal. It may be more relevant for the near future to design a high throughput kidney-on-a-chip with multiple flow channels that can be introduced early into the drug screening process. The results from high throughput kidney-on-a-chip systems can then be applied to the development of scaled human-on-a-chip modules. The focus on only the kidney also has the advantage that early introduction of a single organ chip will allow for easier adoption by both academic and industrial scientists.

CONCLUSION

In summary, the design of a 1:1000 scaled model of the glomerulus and proximal tubule was investigated along with scaling metrics and design parameters. Initial prototyping was performed for the proximal tubule and preliminary data suggest that epoxy glue or compression based bonding should be further pursued. Scaled modeling of the kidney is still in an early stage and many obstacles need to be overcome before an accurate model can be built. A more relevant near term direction to pursue may be high throughput models, which can generate data that can eventually be translated back to scaled models.

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CHAPTER 5:

SUMMARY AND FUTURE DIRECTIONS

Drug testing is a long and expensive process that is hindered by low success rates due, in part, to the limitations of current pre-clinical testing methods. Screening of novel drugs is typically performed on standard two-dimensional cell cultures and in animal models, neither of which accurately reflect the functions of the human body. This shortcoming causes significant attrition of potential drug candidates in the transition from pre-clinical testing to clinical trials in humans due to unanticipated clearance and toxicity concerns[1]. One organ that plays a significant role in drug disposition is the kidney, which filters blood and is responsible for elimination of over one third of all drugs. Inadequate pre-clinical modeling of the kidney accounts for approximately 10-20% of drug candidate withdrawals during clinical trials[2,3]. Additionally, approved drugs can also result in unexpected acute kidney injury, particularly in certain susceptible subsets of the population such as older adults[4]. Therefore, it is important to improve both accuracy and versatility of pre-clinical renal models such that increased clearance and toxicity issues can be identified early and in diverse populations. In this dissertation, this goal of improving *in vitro* modeling of the kidney was approached from three different directions.

The proximal tubules of the kidney are responsible for a majority of drug handling in the kidney and are the most important to model *in vitro*[5]. Therefore, in Chapter 2, a variety of proximal tubule cell models (MDCKs transfected with a pair of human organic cation transporters (hOCT2/hMATE1), SV40 immortalized human proximal tubule cells and hTERT immortalized

human proximal tubule cells) were characterized to determine the strengths and limitations of each and identify the best line for *in vitro* prediction of renal drug disposition. Specifically, cells were assessed on a set of metrics relevant to renal drug handling, including morphology and monolayer formation, gene expression and functionality of organic cation drug transporters, and cell viability and biomarker release after exposure to cisplatin. The results demonstrated that while the human cell lines hold some promise, non-human transfected cell lines remain more robust models of proximal tubule drug transport due to superior monolayer formation, transporter expression and function and ease of handling. When added to the existing body of work in this area, this study clearly demonstrates that more efforts need to be invested in developing cell models that are better able to reproduce *in vivo* proximal tubule functionality. The inability of available human derived cell lines to meet this need suggests that investing in a different source of cells would be beneficial. Stem cell derived proximal tubule cells may be the most appropriate model to pursue for this purpose. Further studies should focus on developing protocols for differentiating stem cells into proximal tubule cells as well as other renal cell types.

Another way to enhance cell functionality *in vitro* is to improve the cellular microenvironment in which they are grown. To address this, the effects of incorporating aspects of *in vivo* physiology, specifically long-term fluid shear stress, into an *in vitro* setting was explored in Chapter 3. Double transfected MDCK cells placed under up to 2 dynes/cm^2 of shear stress showed significant upregulation of both organic cation transport and transporter expression. Furthermore, removal of cilia eliminated the effects of shear on ASP+ transport with no effect on ASP+ transport under static conditions. These results indicate that shear stress affects active transport of organic cations and expression of relevant transporters in renal cells in a cilia dependent manner. Further studies probing the biology underlying the interaction between ciliary

mechano-sensation and transporter function are warranted. Future studies should also explore the effects of shear stress on other types of active transport to investigate whether the same cilia-dependent effect is present. More broadly, the effects of long-term shear stress on human cell lines should be explored to determine if functionality can be improved.

The kidney does not function in isolation in the human body. Other organs impact drug exposure to the kidney and therefore affect renal clearance and toxicity. To account for this, significant efforts are ongoing for the design of human-on-a-chip models that allow for interaction between different organ modules. Chapter 4 of this dissertation focused on designing a 1:1000 scaled kidney-on-a-chip module that can be incorporated into a 1:1000 scaled *in vitro* human system. The proposed design is composed of two segments that mimic the glomerulus and proximal tubule. Each compartment is composed of a parallel plate bioreactor with fluid flow channels mimicking urine and filtrate flow in the kidney and filters providing either a sieving or growth surface. Physical parameters were scaled based on filtration rate for the glomerular segment and water reabsorption for the proximal tubule segment, two critical functions of the kidney. Preliminary efforts at scaling and prototyping revealed significant limitations in the scaling metric used for the proximal tubule, primarily due to the complexity of proximal tubular functionality and limitations of available cell lines. Much more effort needs to be invested in identifying appropriate metrics for scaling and in improving functionality of cell lines. A more relevant near term direction to pursue may be simpler but high throughput models that may represent only limited functionality but can be used for high throughput drug testing. Data generated from developing such systems can eventually be translated back to scaled models.

In summary, this dissertation initiated the development of a more accurate *in* vitro model of the kidney for use in drug clearance and toxicity testing. It offers insight into the limitations of

existing cell models, expands our current knowledge of the interaction between the cellular environment and drug transport functionality and provides a foundation for the future development of a scaled kidney-on-a-chip module.

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