A human proximal tubule-on-a-chip to study renal disease and toxicity

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ABSTRACT

Renal disease is a global problem with unsustainable health-care costs. There currently exists a lack of accurate human renal disease models that take into account the complex microenvironment of these tissues. Here, we present a reusable microfluidic model of the human proximal tubule and glomerulus, which allows for the growth of renal epithelial cells in a variety of conditions that are representative of renal disease states including altered glomerular filtration rate, hyperglycemia, nephrolithiasis, and drug-induced nephrotoxicity (cisplatin and cyclosporine). Cells were exposed to these conditions under fluid flow or in traditional static cultures to determine the effects of a dynamic microenvironment on the pathogenesis of these renal disease states. The results indicate varying stress-related responses (α -smooth muscle actin (α -SMA) expression, alkaline phosphatase activity, fibronectin, and neutrophil gelatinase-associated lipocalin secretion) to each of these conditions when comparing cells that had been grown in static and dynamic conditions, potentially indicating more realistic and sensitive predictions of human responses and a requirement for a more complex “fit for purpose” model.

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I. INTRODUCTION

Renal disease is a worldwide health burden resulting in millions of deaths every year. The Centers for Disease Control and Prevention estimates that 1 in 10 adults in the United States may have chronic kidney disease (CKD), which is often accelerated by factors such as hypertension (found in 28% of cases) and diabetes (44% of cases) (Dourish and Dawson, 2014). Studying the onset of renal disease can be challenging, as current *in vitro* culturing methods lack the physiological stimuli and architecture of these tissues *in vivo*. A number of studies have shown that the tissue microenvironment plays major roles in healthy renal cell physiology and function (Duan et al., 2008; Essig and Friedlander, 2003; Fisher et al., 2001; and Raghavan et al., 2014), but this environment may also be highly influential in disease progression and the study of drug efficacy to treat these disease conditions. To overcome some of the drawbacks of *in vitro* cell culture, animal models are used to study diseases. Animal studies have allowed for a greater understanding of disease mechanisms, but their value in predicting the drug efficacy for disease treatment and

prevention remains questionable (Becker and Hewitson, 2013). Additionally, the combination of artificially induced disease, and laboratory and handling conditions can significantly impact behavior and disease pathologies, adding variables that may confound data (Akhtar, 2015). Organ-on-a-chip systems are a relatively new technology that may help overcome some of these concerns by bridging the gap between *in vitro* cell-based studies and *in vivo* animal models. Through the use of these models, human cells can be grown in a more physiologically relevant environment, potentially leading to more realistic cell behaviors and responses to new therapeutics or treatment regimens in diseased conditions.

Many disease states fall under the category of CKD, and previous *in vitro* and *in vivo* studies have investigated these conditions. Diabetic nephropathy is a serious complication of diabetes and can account for up to 40% of cases of end-stage renal disease (Forbes et al., 2007). Hyperglycemia induced by diabetic conditions is one of the most important risk factors in the progression of tubular fibrosis and excess extracellular matrix (ECM) deposition in the kidney. These high glucose

levels can be directly tubulotoxic, but it has recently been shown that these conditions may also stimulate elevated expression of fibrogenic cytokine TGF- β (Sharma *et al.*, 1996), α -SMA expression, and additional ECM deposition (Lee and Ha, 2005 and Yamagishi *et al.*, 2007). Nephrolithiasis, or renal calculi, more commonly referred to as kidney stones, usually consist of calcium oxalate monohydrate (COM), which accounts for the composition of roughly 80% of stones (Finkelstein and Goldfarb, 2006). Finally, there is a great need for a realistic model of drug-induced nephrotoxicity. Renal toxicity is one of the most widely reported adverse effects during drug development, and it can only be detected late into the drug development process (Jang *et al.*, 2013 and Tiong *et al.*, 2014). In this study, alkaline phosphatase (AP) activity, neutrophil gelatinase-associated lipocalin (NGAL) secretion, and fibronectin secretion were used as indicators of cytotoxicity. AP is an enzyme that is normally expressed in renal tissues; however, altered levels of this enzyme can be an indication of disease or cell distress (Prasad *et al.*, 2005). NGAL is an emerging biomarker for acute kidney injury (AKI), representing structural damage without apparent functional injury, and it is one of the most highly induced proteins in the tubule after acute kidney injury (AKI) in animal models (Devarajan, 2010). Finally, fibronectin deposition has been associated with renal cell injury and fibrosis in almost all progressive chronic kidney diseases (Liu, 2011). While animal models and static culture of human cells are the current gold standards in toxicity testing, a more realistic model may be necessary to test the drug efficacy and physiological responses in humans.

Tubular shear stress has been shown to affect the phenotype of renal epithelial cells, leading to changes in the actin cytoskeleton and brush border (Duan *et al.*, 2008 and Essig and Friedlander, 2003). Additionally, shear stress has been shown to enhance cell polarization and cilia formation, and this effect is further enhanced when cells are grown upon porous substrates (Jang *et al.*, 2013). While the effects of shear stress on healthy cell function have been investigated, studies on the effects of a dynamic environment on disease progression and treatment are currently lacking. The use of cell lines can often be advantageous because of their homogeneous and immortalized populations; however, these cells can lose their key phenotypic characteristics, leading to altered behaviors. It is essential to choose an appropriate cell line when investigating drug-induced nephrotoxicity and renal disease. Previous studies have demonstrated that HK-2 cells offer a suitable model for *in vitro* toxicity studies. This cell line expresses many of the same key transporters and demonstrates similar toxic responses to those of renal proximal tubule cells *in vivo* (Chang *et al.*, 2016 and Ryan *et al.*, 1994). HK-2 cells support clinical data in humans, for example, HK-2 studies with ifosfamide and acyclovir showed varying clinical nephrotoxic patterns (Gunness *et al.*, 2010).

In this study, HK-2 cells were grown within a multi-layered, reusable microfluidic device that has previously been shown to mimic some of the functions of the human proximal tubule and glomerulus (Sakolish and Mahler, 2017). The major benefits of this device are the multiple chambers (allowing for

the study of uptake and transport) and the reusability of this device, which is a rare trait in microphysiological systems. These devices are milled from polycarbonate, and they can be sterilized via autoclave to reuse over many cycles. Physiologically realistic shear stress (0.2–0.8 dyn/cm²) (Essig *et al.*, 2001; Kim and Takayama, 2015) was provided to the HK-2 proximal tubule cultures within devices. Cells were exposed to conditions that are representative of altered glomerular filtration rate (GFR), hyperglycemia, nephrolithiasis, and drug-induced nephrotoxicity to study differences between traditional culturing and cells grown within this microphysiological device. After exposure to these conditions, α -SMA expression was quantified in addition to AP activity, fibronectin, and NGAL release in the culture effluent.

II. MATERIALS AND METHODS

A. Microfluidic device fabrication and assembly

The microfluidic device used for this work was previously used to culture HK-2 cells and model the filtration and reuptake of compounds in primary urine (Sakolish and Mahler, 2017). A detailed description of the device fabrication and assembly is available in the [supplementary material](#).

In addition to the microfluidic device described above, which serves as a growth platform for the proximal tubule cells, a small chamber was added into the top channel circuit to act as a passive glomerular filtration barrier [Fig. 1(a)]. A stainless-steel filter housing (14 mm diameter, Advantec MFS, Inc.) was fitted with an 8 nm pore size (50 000 MWCO) polyethersulfone (PES) membrane (Sartorius, #14650-76-D). The medium flows through this chamber prior to entering the top fluidic channel of the proximal tubule device, removing large molecular weight particles and proteins and creating a more realistic “primary urine.” This filter was not added to the bottom fluidic channel, which acted as a vascular component.

B. Cell culture

1. Static

HK-2 cells were seeded on fibronectin-coated (8 μ g/cm², Corning) 0.4 μ m pore size polycarbonate Transwell membranes (Corning) in a 6-well culture plate at a density of 100 000 cells/cm² and allowed to attach overnight prior to experimentation. These cells were incubated in DMEM/F12 (Thermo Fisher Scientific) with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific) at 37 °C and 5% CO₂.

2. Fluidic

HK-2 cells were seeded onto polycarbonate membranes (0.4 μ m pore size, Whatman) with a fibronectin coating (8 μ g/cm²) in 6 well growth plates at a density of 100 000 cells/cm², and allowed to adhere overnight in DMEM/F12 medium with 10% FBS at 37 °C and 5% CO₂. After this incubation period, membranes were removed from the 6 well plates and placed within the polycarbonate devices. All necessary tubing and device reservoirs were perfused with DMEM/F12 culture

medium for 15 min to remove air bubbles, then connected to the inlets of the top and bottom channels. The medium was flowed through the devices and tubing for several hours to remove bubbles. Once the devices were primed with medium, the remaining tubing was connected to the outlets to seal the system, and flow was initiated. Two different device configurations were used for the experiments, the proximal tubule device either with or without the attached glomerulus filter. Due to the restrictive flow through the 8 nm glomerular filter, the resulting shear stress over the proximal tubule cells was reduced in the models containing the glomerulus. Shear stress rates in the proximal tubule device were determined by adding fluorescent microbeads (100 nm diameter) to the top chamber of the proximal tubule device and measuring bead movement through the channels with a fluorescent microscope (Olympus IX51 with a Hamamatsu ORCA-05G camera). These videos were then analyzed using ImageJ (imagej.nih.gov/ij/) and the manual tracking plugin as previously described (Sakolish and Mahler, 2017). Flow through the glomerular filter was determined by weighing the filtered effluent in 15-min increments over 1 h.

Table I describes the experimental conditions including the flow rate, the presence or absence of the glomerular filter, and approximate filtration pressure across the glomerulus membrane for each condition tested. The pressure drop across the glomerular filtration membrane was calculated using the Hagen–Poiseuille equation as described by Kanani et al. (2010) [Eq. (1)]:

$$\Delta P = \left(\frac{Q}{A} \right) / \left(\frac{\epsilon^* r_p^2}{8 \mu^* \delta} \right) \quad (\text{Pa}), \quad (1)$$

where pressure drop, or filtration pressure, ΔP is calculated across the glomerulus membrane in Pa, Q is the flow rate in m^3/s , A is the cross-sectional flow area in m^2 , ϵ is the membrane porosity, r_p is the pore radius in meters, μ is the viscosity in Pa s, and δ is the membrane thickness in meters. To estimate the pressure drop across the PES membranes, a flow rate of $4.78 \mu\text{l}/\text{min}$ ($7.97 \times 10^{-11} \text{ m}^3/\text{s}$, measured experimentally), cross-sectional flow area of 1.54 cm^2 (0.000154 m^2), porosity of 4.5% (Dietz et al., 1992), pore radius of 4 nm ($4 \times 10^{-9} \text{ m}$), viscosity of 0.00089 Pa s (viscosity of water), and membrane thickness of $120 \mu\text{m}$ ($1.2 \times 10^{-4} \text{ m}$) were used for

TABLE I. Experimental conditions in the static controls, proximal tubule device, or proximal tubule device with glomerular filtration.

Experimental conditions	Glomerular filter	Proximal tubule τ (dyn/cm ²)	Glomerular filtration ΔP (mm Hg)
Static Transwell	No	N/A	N/A
Physiological shear, calcium oxalate exposure	No	0.8	N/A
Altered glomerular filtration rate (high shear)	No	5	N/A
High glucose, drug exposure	Yes	0.2	4605

calculations. Based on these calculations, the filtration pressure across the glomerular filter is 614 kPa or 4605 mm Hg. This is much higher than the physiologically normal filtration pressure of 10 mm Hg (Bohle et al., 1998) but within the nanofiltration operating pressure range (National Research Council, 1997).

C. Immunocytochemistry

A detailed immunocytochemistry protocol for detection of α -SMA protein expression is available in the [supplementary material](#).

D. Detection of biomarkers

A detailed protocol for AP, NGAL, and fibronectin detection is available in the [supplementary material](#).

E. Modeling of altered GFR

Cells were seeded on fibronectin-coated membranes and allowed to grow for 24 h prior to exposure to shear stress. To investigate the effects of altered shear stress, cells were exposed to either static, physiological shear ($0.8 \text{ dyn}/\text{cm}^2$), or high shear ($5 \text{ dyn}/\text{cm}^2$) for 2 h within the microfluidic device in a configuration lacking the glomerular filter (which would have restricted flow rate). After this exposure period, cells were fixed and stained for the presence of α -SMA. Additionally, the cell lysate was collected to determine the degree of AP activity, normalized to the total protein concentration following manufacturer's protocols. Finally, the effluent from devices and media from static wells was collected to determine the concentrations of excreted NGAL and fibronectin.

F. Modeling of hyperglycemia

To model hyperglycemia, cells were adapted to serum-free keratinocyte-SFM (baseline 8mM glucose, Thermo Fisher Scientific) over 3 passages, and seeded in Transwells (static) or devices (fluidic, $0.2 \text{ dyn}/\text{cm}^2$) then exposed to either control (8mM glucose) or high-glucose (30mM glucose) SFM for 12 h. The baseline glucose of DMEM/F12 was too high to use for this study ($\sim 18 \text{ mM}$). After exposure, cells were fixed, and α -SMA protein expression was quantified through immunocytochemistry. Additionally, AP activity and the presence of NGAL and fibronectin in the resulting culture media from all treatment conditions was monitored using commercial kits and normalized to total cell protein using the Bradford assay.

G. Calcium oxalate exposure

Calcium oxalate crystals were created following a protocol adapted from Sun et al. (2015). A detailed protocol is available in the [supplementary material](#). Scanning electron microscopy indicated that the crystals are roughly $3 \mu\text{m}$ in diameter [Fig. 4(a)], and their composition was confirmed through the use of energy dispersive spectroscopy (EDS; EDAX, Ametek),

which indicated characteristic ratios of oxygen and calcium (see Fig. 1 in the [supplementary material](#) for EDS results).

Crystals were added to cell-seeded Transwells or device membranes at a concentration of 0.3 mg/ml in Keratinocyte-SFM. Static and fluidic (0.8 dyn/cm^2) cultures were incubated with these crystals for 2 h within devices lacking a glomerular filter (due to the size restrictive nature of the filter, preventing the passage of crystals), then cultures were washed with phosphate buffered saline (PBS) to remove unassociated crystals. α -SMA protein expression was monitored via immunocytochemistry, and AP activity, NGAL, and fibronectin concentration were quantified and normalized to total cell protein.

H. Drug-induced toxicity

Cells were seeded on fibronectin-coated Transwell membranes in 6-well plates at a density of $100,000 \text{ cells/cm}^2$ and allowed to proliferate overnight in DMEM/F12 medium. After this growth period, membranes were exposed to cisplatin (1.4 mM, Sigma-Aldrich, USP standard), cyclosporine (0.27 mM, Sigma-Aldrich, USP standard), vehicle solution (0.33% v/v ethanol in DMEM/F12), or control medium in static conditions over a period of 24, 48, and 72 h. After this exposure period, membranes were treated with Calcein-AM live stain (Thermo Fisher) to determine the viability of treated cells relative to controls. Viability levels were determined by measuring the total fluorescence with a Synergy 2 fluorescent plate reader (BioTek) and a 485/528 Excitation/Emission filter.

To determine the effects of shear stress on toxic responses, cells were seeded in either static plates or devices (0.2 dyn/cm^2 shear stress) and exposed to cisplatin (1.4 mM), cyclosporine (0.27 mM), vehicle solution (0.003% v/v ethanol in DMEM/F12), or control DMEM/F12 medium for 24 h. Data on AP activity, total protein levels (Bradford), α -SMA expression, and the concentrations of NGAL and fibronectin in the medium were collected after this 24-h exposure period.

I. Statistical analysis

The data presented in this study are expressed as mean $\pm$ SEM. One-way ANOVA with Tukey's post-test was used as an assessment between multiple groups (groups that do not share letters have a statistically significant difference between means). An unpaired Student's *t*-test was used to assess statistical differences between two conditions. The sample size is indicated by *n*, where sample size is representative of the total number of independent sample collections. Statistical analyses were conducted using Graphpad Prism 5 (GraphPad Software, San Diego, CA).

III. RESULTS

A. Effects of the glomerular filter

After cells were grown under a steady fluid shear stress of 0.2 dyn/cm^2 for 5 h in devices with or without a glomerular filter attachment, effluent samples were collected. NGAL [Fig. 1(b)] and fibronectin [Fig. 1(c)] concentration was measured and normalized to total cell protein. While there was a decrease in NGAL secretion with the devices that had glomerular filtration, this change was not significant (No filter: 0.67 ± 0.08 , Devices with filter: $0.41 \pm 0.07 \text{ ng NGAL/mg total protein}$). However, fibronectin was detected in significantly lower quantities from the effluent of devices with the glomerular filter (No filter: 127.60 ± 24.03 ; Devices with filter: $36.97 \pm 18.78 \text{ ng fibronectin/mg total protein}$).

B. Model of altered GFR

After a 2-h exposure period to static, physiological (0.8 dyn/cm^2), or hypertensive (5 dyn/cm^2) conditions, cells were stained for the fibrotic marker α -SMA [Figs. 2(a)–2(c)]. It was found that cells grown in static conditions expressed small amounts of α -SMA ($5.3 \pm 0.9\%$ positive staining per field),

FIG. 1. (a) Device configuration highlighting the glomerular filter attachment. Device cross section is shown to highlight tubular and vascular compartments. (b) NGAL secretion ($n = 3$), and (c) fibronectin secretion ($n = 3$) normalized total protein. Unpaired Student's *t*-tests were used to determine the significance between conditions (* $p < 0.05$).

FIG. 2. Effects of shear stress on α -SMA expression. Cells were seeded into microfluidic devices and exposed to steady shear stress for 2 h. Cells were fixed and stained for α -SMA expression following (a) static, (b) physiological (0.8 dyn/cm²), and (c) high (5 dyn/cm²) steady shear stress. Scale bars = 100 μ m. The results indicate a correlation between the amplitude of shear stress and the degree of α -SMA expression. (d) Quantitative results of α -SMA expression under increasing shear rates. The results are shown as % positive staining per field, mean $\pm$ SEM, n = 6. Unpaired Student's *t*-tests were used to compare differences between means. ***p* < 0.01, ****p* < 0.005, *****p* < 0.001.

and that this expression increased significantly at each step increase in shear stress [0.8 dyn/cm²: 22.1 $\pm$ 2.9% and 5 dyn/cm²: 35.7 $\pm$ 3.9% positive staining per field, Fig. 2(d)].

C. Model of hyperglycemia

To model hyperglycemia, cells were grown in static or fluidic conditions (0.2 dyn/cm²) in either a control, serum-free, low glucose Keratinocyte-SFM (8mM), or in glucose-treated SFM (30mM) for 12 h. To monitor fibrosis and cell distress, respectively, staining for α -SMA and monitoring of AP activity, and NGAL and fibronectin secretion was performed on these cell cultures. α -SMA expression increased significantly in fluidic cultures compared to controls, but there was no significant change observed between static cultures [static

control: 7.2 $\pm$ 0.5; static treatment: 8.6 $\pm$ 0.3; fluidic control: 4.5 $\pm$ 0.9; and fluidic treatment: 8.5 $\pm$ 1.6% positive staining per field, Fig. 3(a)]. NGAL secretion was significantly different between static and fluidic controls, as well as static and fluidic treatments, but there was no significance between treatments and their paired controls [static control: 11.5 $\pm$ 0.5; static treated: 17.4 $\pm$ 6.4; fluidic control: 0.9 $\pm$ 0.1; and fluidic treatment: 1.5 $\pm$ 0.9 ng NGAL/mg total protein, Fig. 3(b)]. Changes in fibronectin secretion were determined to be non-significant between controls and treatments; however, control cells grown in fluidic conditions appeared to secrete a significantly lower amount of fibronectin than static controls [static control: 334.7 $\pm$ 2.3; static treated: 226.6 $\pm$ 64.4; fluidic control: 109.3 $\pm$ 39.6; and fluidic treatment: 93.3 $\pm$ 17.8 ng fibronectin/mg total protein, Fig. 3(c)]. Finally,

FIG. 3. Effects of high glucose exposure on renal distress markers. HK-2 cells were adapted to low glucose (8mM) culture medium for 3 passages and then exposed to high glucose (30mM) medium for 12 h. (a) α -SMA protein (n = 9), (b) NGAL (n = 3), (c) Fibronectin (n = 3), and (d) alkaline phosphatase (AP) (n = 3) activity. Under fluidic conditions, cells were exposed to 0.2 dyn/cm² steady shear stress. The results are shown as mean $\pm$ SEM. An unpaired Student's t-test was used to compare differences between means (*p < 0.05, **p < 0.01, ****p < 0.001).

no significant changes in AP activity were observed under any culturing conditions [static control: 0.25 ± 0.13 ; static treated: 0.39 ± 0.09 ; fluidic control: 0.25 ± 0.15 ; and fluidic treated: 0.47 ± 0.19 μ g AP/mg total protein, Fig. 3(d)].

D. Calcium oxalate exposure

Calcium oxalate monohydrate (COM) crystals were added at a concentration of 0.3 mg/ml to static and fluidic (0.8 dyn/cm²) cultures for a treatment period of 2 h. To monitor fibrosis and cell distress, staining for α -SMA, measurement of NGAL, fibronectin secretion, and AP activity was performed on these cultures. It was determined that static cultures incubated with COM crystals had a higher degree of fibrosis as indicated by a rise in α -SMA expression [Fig. 4(b)], though this increase was not statistically significant (static control: 4.5 ± 0.4 ; static treatment: 9.7 ± 2.6 % positive staining per field). Fluidic cultures exposed to COM crystals appeared relatively unaffected, as α -SMA expression fell within the same range as controls (fluidic control: 4.2 ± 0.5 ; fluidic treatment: 3.7 ± 0.6 % positive staining per field). NGAL secretion [Fig. 4(c)] was significantly increased between static controls and treatments, and while this same trend was observed in fluidic conditions, this change was not significant (static control: 0.22 ± 0.01 ; static treatment: 0.62 ± 0.12 ; fluidic control: 0.19 ± 0.02 ; and fluidic treatment: 1.2 ± 0.5 ng NGAL/mg total protein). In the case of fibronectin secretion [Fig. 4(d)], while no significance was observed between static controls and treatments, fibronectin secretion

was found to be significantly higher in fluidic cultures compared to fluidic controls (static control: 132 ± 7 ; static treatment: 159 ± 9 ; fluidic control: 133 ± 7 ; and fluidic treatment: 258 ± 15 ng fibronectin/mg total protein). Finally, it was observed that in the case of AP activity [Fig. 4(e)], while COM-treated static cultures did have significantly higher AP levels than their controls (static control: 1.1 ± 0.2 and static treatment: 2.6 ± 0.2 μ g AP/mg total protein), fluidic cultures exhibited a much greater (14-fold) increase in the amount of AP present in cell lysate (fluidic control: 1.1 ± 0.1 , COM fluidic: 15.4 ± 3.7 μ g AP/mg total protein).

E. Drug-induced toxicity

To model pharmaceutical nephrotoxicity, cultures were exposed to cyclosporine (0.33 mg/ml, 0.27mM) or cisplatin (0.41 mg/ml, 1.4mM). To monitor viability over time, static cultures were treated with these compounds for 24, 48, or 72 h to determine the length of time necessary to induce significant changes in cell viability. These exposure concentrations were chosen to reflect renal exposure based off of human serum steady state values [cyclosporine: 333 ng/ml (Atkinson et al., 1984); cisplatin: 414 ng/ml (Urien and Lokiec, 2004)] with factors of 10 added to reflect the differences in exposure periods (hours in culture vs days/weeks in patients), cell source (cell line vs human tissues in vivo), and the differences in concentration between human serum and urine precursor (which is highly concentrated in the nephron). While the

FIG. 4. Effects of calcium oxalate monohydrate (COM) exposure on renal distress markers. Static and fluidic (0.8 dyn/cm^2) cultures were exposed to crystals for 2 h. (a) Scanning electron microscopy (SEM) image of calcium oxalate crystals demonstrating a diameter of approximately $3 \mu\text{m}$. Scale bar = $1 \mu\text{m}$. (b) Expression of α -SMA (results are shown in % positive staining for α -SMA per field, $n=4$). (c) NGAL secretion ($n=3$), (d) Fibronectin ($n=3$), and (e) alkaline phosphatase (AP) ($n=4$). Unpaired Student's t -tests were used to determine the significance between treatments and controls (* $p < 0.05$, ** $p < 0.01$).

exposure concentrations used in this study were much higher than a typical human serum concentration, these values were chosen to test the system as a proof of concept study and were confirmed to elicit a response in static culture HK-2 cell viability within the short exposure period. It was found that after 24 h of static culture, these nephrotoxins significantly reduced cell viability as indicated through Calcein-AM fluorescent live staining, and that cell viability was further reduced after 48 and 72 h in a dose- and time-dependent manner [Fig. 5(a)]. At every time point, cells treated with cyclosporine or cisplatin had significantly lower viability than controls, with cisplatin acting as a positive control. Additionally, it should be noted that the ethanol vehicle (control for cyclosporine) was not significantly different than untreated controls (except after 72 h), indicating that decreases in viability were likely due to the cyclosporine rather than its vehicle.

To determine the effects of shear stress on cytotoxicity, cells were grown in either static or fluidic (0.2 dyn/cm^2) conditions within devices in the presence of cisplatin (1.4 mM), cyclosporine ($270 \mu\text{M}$ in 0.33% ethanol) or 0.33% ethanol (as a vehicle control for cyclosporine) for a period of 24 h. After exposure, the AP activity was quantified and normalized to total protein in these cultures [Fig. 5(b)]. It was found that AP

activity was significantly increased in fluidic cultures compared to static cultures that had been treated with the same compound for both cyclosporine (static cyclosporine: 2.6 ± 0.3 and fluidic cyclosporine: $6.0 \pm 1.2 \mu\text{g AP/mg total protein}$) and cisplatin (static cisplatin: 3.3 ± 0.3 and fluidic cisplatin: $5.8 \pm 0.5 \mu\text{g AP/mg total protein}$), but control values were not significantly different between static and fluidic culturing conditions (static control: 2.1 ± 0.1 and fluidic control: $1.8 \pm 0.2 \mu\text{g AP/mg total protein}$). The ethanol vehicle did not appear to have any significant effects on AP activity in static or fluidic cultures (static vehicle: 1.1 ± 0.2 and fluidic vehicle: $1.3 \pm 0.1 \mu\text{g AP/mg total protein}$).

In addition to monitoring AP activity, NGAL and fibronectin concentration was measured in the effluent of devices and medium in static wells then normalized to total protein in each sample. While the vehicle did not have an effect on AP activity, significant changes between control and vehicle were observed through changes in fibronectin secretion [static control: 394 ± 23 ; static treatment: 248 ± 22 ; fluidic control: 274 ± 14 ; and fluidic treatment: $355 \pm 16 \text{ ng fibronectin/mg total protein}$, Fig. 5(c)]. No significant changes between controls and treatments were observed in NGAL secretion, though it was noted that NGAL was found in lower concentrations in fluidic cultures [static control: 0.62 ± 0.03 ; static treatment:

FIG. 5. Effects of pharmaceutical compounds on cell viability. (a) 24 h after seeding, cells in static cultures were exposed to cisplatin, cyclosporine, or vehicle control and then stained with calcein-AM live stain to determine the effects on cell viability. The results show mean $\pm$ SEM, $n = 3$ using a one-way ANOVA with Tukey's *post hoc* testing. (b) Static and fluidic (0.2 dyn/cm^2) cultures were exposed to (c) and (d) vehicle, (e) and (f) cyclosporine (0.33 mg/ml), or (g) and (h) cisplatin (0.414 mg/ml). NGAL and fibronectin concentrations were measured in the medium from plates and the effluent from devices and are shown as mean $\pm$ SEM, unpaired Student's *t*-tests were used to compare controls and treatments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

0.56 ± 0.21 ; fluidic control: 0.39 ± 0.04 ; and fluidic treatment: $0.90 \pm 0.48 \text{ ng NGAL/mg total protein}$, Fig. 5(d)]. When cells were exposed to cyclosporine, no significant changes in fibronectin secretion were observed between treatments and their

individual controls [static control: 394 ± 23 ; static treatment: 308 ± 25 ; fluidic control: 274 ± 14 ; and fluidic treatment: $581 \pm 100 \text{ ng fibronectin/mg total protein}$, Fig. 5(e)]; however, a significant increase in NGAL secretion was observed between fluidic control and treatment values, with no major changes under static conditions [static control: 0.62 ± 0.03 ; static treatment: 0.75 ± 0.19 ; fluidic control: 0.39 ± 0.03 ; fluidic treatment: $0.66 \pm 0.06 \text{ ng NGAL/mg total protein}$, Fig. 5(f)]. Finally, upon exposure to cisplatin, fibronectin [static control: 394 ± 23 ; static treatment: 419 ± 54 ; fluidic control: 274 ± 14 ; and fluidic treatment: $472 \pm 27 \text{ ng fibronectin/mg total protein}$, Fig. 5(g)] and NGAL secretion [static control: 0.62 ± 0.03 ; static treatment: 0.67 ± 0.04 ; fluidic control: 0.39 ± 0.04 ; and fluidic treatment: $1.04 \pm 0.03 \text{ ng NGAL/mg total protein}$, Fig. 5(h)] was significantly increased in fluidic cultures only, with minimal effect on static cultures.

IV. DISCUSSION

In this study, four of the most common renal disease states including altered GFR, hyperglycemia, kidney stones, and drug-induced nephrotoxicity were modeled using a reusable microfluidic device platform, and compared against traditional static cultures that had been treated in parallel. Significant differences in cell responses were observed between these two culturing techniques. We therefore propose that growing cells within these microfluidic devices may lead to more realistic cell/tissue responses, potentially allowing for a better prediction of human responses. Other groups have previously studied the effects of shear stress on proximal tubule cells (Duan et al., 2008; Essig and Friedlander, 2003; Jang et al., 2011,2013; Vedula et al., 2017; and Weber et al., 2016); however, many of these microfluidic platforms lack a second “endothelial” channel, to allow for the study of tubular reabsorption/secretion and transport, which are major functions of these tissues. While this device does not necessarily recapitulate the 3D tubular structure and curvatures of the human proximal tubule, it retains many of the essential elements of microphysiological systems (fluid flow, low fluid-tissue ratios, porous growth substrate, and individual control over flow in cell and “vascular” channels), while being general enough to adapt to other barrier tissues in future studies. Additionally, to our knowledge, this is the first example of a proximal tubule device that considers the glomerulus—an essential barrier tissue prior to the proximal tubule, which can be involved in many disease conditions in the kidney. Lastly, we believe that the reusability of this device makes it unique in the tissue chip space.

Here, we found that distress markers NGAL and fibronectin decreased in cultures that were grown within microfluidic devices that contained a glomerular filter. The medium has to pass through this filter attachment before reaching the cell seeded chamber, removing many of the serum proteins from the cell culture medium that can often cause proteinuria-like conditions with kidney cells grown *in vitro*. This pre-filtration is likely the cause for the reduction in the secretion of these markers; therefore, we attempted to use

the filter attachment in as many of the experiments as possible (with the exception of increased GFR and calcium oxalate crystal exposure due to restrictions in flow rate and size exclusion, respectively).

Altered GFR can lead to many downstream renal diseases if left untreated. Alterations in blood pressure can result in changes to filtration rate, thereby raising the glomerular filtration rate (GFR), and increasing shear stress in the proximal tubules. This can lead to stretching of vessel walls and renal tubular fibrosis. Fibrosis is characterized by an excessive accumulation and deposition of ECM. This process occurs as a reparative reaction to cell injury, ultimately leading to tissue scarring and loss of function if left untreated (Birbrair *et al.*, 2014 and Essig and Friedlander, 2003). To model fibrosis in our device, we grew cells on membranes at static, physiological (0.8 dyn/cm²) (Kim and Takayama, 2015), and hypertensive (5 dyn/cm²) shear stress (Grabias and Konstantopoulos, 2012) and monitored α -SMA expression in each of these respective culturing conditions. Previous studies have identified α -SMA expression as a factor that accelerates contraction of the tubulointerstitial tissues, leading to renal atrophy and failure (Ina *et al.*, 2011); however, the effects of shear stress on α -SMA have not been extensively studied. In our experiments with HK-2 cells, it was observed that the expression of α -SMA was highly correlated to shear stress levels. The results indicated that cells grown in static conditions had relatively low expression of α -SMA and that the introduction of physiological shear stress increased expression levels more than 4-fold. This increase in α -SMA expression was expected in fluidic cultures because α -SMA is also responsible for cell adhesion, structure and integrity in healthy cells (Dominguez and Holmes, 2011). The introduction of shear stress to cultures puts strain on these cells, requiring greater actin involvement for adhesion. However, a further increase from the observed physiological levels of α -SMA expression could potentially be an indicator of fibrosis in these cells. When cells were exposed to a further increased shear stress of 5 dyn/cm², α -SMA expression was increased another 1.6-fold from physiological levels, and this pattern is expected to continue as shear stress is further increased. However, due to restrictive flow within the device, this was the maximum shear stress that was achievable with this system. These results indicate that tubular fibrosis may in part be dependent on mechanical factors such as glomerular filtration rate.

To model hyperglycemia within these platforms, cells were adapted to low glucose medium (8mM glucose) and then either exposed to untreated serum-free control medium (SFM containing a baseline of 8mM glucose), or a high-glucose adjusted SFM (30mM glucose). Healthy blood-glucose levels are up to 7.5mM, while hyperglycemia is characterized as being greater than 11.1mM (Umpierrez *et al.*, 2002). High concentrations of glucose have previously been shown to induce fibrosis in kidney cells through changes in cell morphology, a loss of cell-cell contacts, and increased α -SMA expression (Hsieh *et al.*, 2012); however, the effects of shear stress on glucose-induced fibrosis have not been thoroughly investigated.

To determine the potential effects of fluid shear stress on hyperglycemic fibrosis, cells were exposed to either a control or high-glucose treated SFM for 12 h in static or physiological shear stress (0.8 dyn/cm²) (Essig *et al.*, 2001). The expression of α -SMA was quantified in static and fluidic high-glucose cultures compared against cells grown in control untreated medium. The results indicated that in static conditions, while the expression of α -SMA did slightly increase in treated cultures compared to the control untreated cells, this increase was not statistically significant. However, after the addition of shear stress, an almost 2-fold increase in the amount of the fibrotic marker α -SMA was expressed compared to fluidic controls. Additionally, AP activity, and NGAL and fibronectin secretion were monitored in these cultures. In experiments with high glucose, there were increases in AP activity and NGAL secretion from control to treated conditions; however, these increases were not significant (though there were significant differences between static and fluidic controls). Fibronectin secretion decreased with high glucose treatments when compared to controls, but this difference was also determined to be non-significant. These results indicated that while the expression of some fibrotic markers such as α -SMA may increase, AP, NGAL, and fibronectin secretion appears to be mostly unaffected by exposure of HK-2 cells to high-glucose medium over this exposure period. However, it is important to note that the base control medium used in this experiment had a glucose concentration of 8mM, which falls just above a normal healthy glucose value that would be found in serum. It is possible that the effects of glucose on these cells under fluid shear stress would be more evident if a lower glucose medium was used as a control or over a longer exposure period. Future studies will need to be more targeted to demonstrate the effects of shear on hyperglycemia, as the results of this study are inconclusive. However, these limited results may indicate that renal fibrosis is not only caused by chemical factors but can also be compounded by mechanical influences such as shear stress, leading to more sensitive responses to altered cell growth conditions.

The effects of shear stress on kidney stone cytotoxicity were also investigated. In a study by Verkoelen *et al.* (1995), the effects of COM crystal association with Madin-Darby canine kidney (MDCK) cells (a canine distal tubule cell line) were investigated. It was found that crystals initially attached to the surface of cell monolayers, but they were later taken up and subsequently eliminated. This process of elimination was carried out to a certain degree without cell injury; however, cells reached a saturation point and could no longer transport the attached crystals. This accumulation has previously been shown to lead to fibrotic responses (Khan, 1995 and Tsujihata, 2008). Schepers *et al.* (2003) demonstrated this internalization through confocal imaging, and they had shown that crystals were located on the surface, as well as inside of proximal tubule cells. Finally, an increase in inflammatory markers and ECM deposition in response to calcium oxalate crystals has previously been demonstrated in MDCK cells by Yamate *et al.* (1999). As we and others have previously identified, transport activity in these cells is highly influenced

through mechanotransduction induced by shear stress (Duan *et al.*, 2008; Essig and Friedlander, 2003; Fisher *et al.*, 2001; Raghavan *et al.*, 2014; and Sakolish and Mahler, 2017). Therefore, the effects of calcium oxalate stones on static and fluidic cell cultures were investigated, resulting in some interesting observations. First, the expression of the fibrotic marker α -SMA was measured in static and fluidic cultures that had been exposed to the control medium or a COM crystal solution (3 μ m diameter, 0.3 mg/ml in SFM) for 2 h. It was observed that in exposed static cultures, there was a non-significant increase in the expression of α -SMA compared to controls. Fluidic cultures did not show any differences in α -SMA expression between treated and untreated cultures. However, when we investigated AP activity, NGAL, and fibronectin secretion, significant differences between control and treated cells were evident. In static cultures, AP activity was significantly increased after treatment with COM crystals; however, this increase is significantly lower than the nearly 14-fold increase in AP shown in fluidic cultures between control and treated cells. NGAL only showed significant increases following COM crystal exposure in static cultures, while fibronectin was significantly higher only in fluidic conditions. Clearly, there are differences in cell responses to calcium oxalate crystals between traditional static culturing and cells grown within the fluidic microphysiological system. We hypothesize that in static cultures, more of the crystals settled onto the surface of monolayers and were therefore able to adhere to the surface of cells, overloading the cells' ability to eliminate the crystals. This may have led to scarring or fibrosis on the apical surfaces of these cell monolayers, causing the observed increase in α -SMA expression. In fluidic cultures however, it is likely that fewer crystals would adhere to monolayers due to the shear stress in the fluidic channel. Under exposure to fluid flow, we expected to see a higher degree of internalization of these crystals due to increases in active transport brought on by mechanotransduction. Therefore, we hypothesize that α -SMA may be a potential marker for external crystal adherence, and AP activity may be an indicator of cell distress brought on by crystal internalization. The results of these studies on renal disease in static and fluidic culturing conditions indicate that a dynamic culturing environment may be necessary to recapitulate realistic cell responses to disease conditions *in vitro*. However, there have also been many recent studies investigating the influences of shear stress on drug-induced nephrotoxicity within microfluidic devices (Jang *et al.*, 2013 and Kim *et al.*, 2016).

In the current state of pharmaceutical studies, there is a 93% attrition rate when transitioning from pre-clinical models to human clinical trials (Nasioudis and Witkin, 2015). There are many factors that contribute to this high attrition rate, but unexpected toxicity is a major factor that may be avoidable if more realistic *in vitro* models are applied. In this model of nephrotoxicity, cyclosporine (Ryffel and Mihatsch, 1986) and cisplatin (Townsend *et al.*, 2003) were chosen because they are both well studied nephrotoxins that cause nephrotoxicity in their original, parent form. Additionally, these pharmaceuticals are small molecules that can pass

through the glomerular barrier unimpeded, making them excellent models to study within this *in vitro* glomerulus and proximal tubule device. The effects of cisplatin and cyclosporine on HK-2 cells have previously been investigated with mixed results (Huang *et al.*, 2015; Kim *et al.*, 2014; Puigmule *et al.*, 2009; and Sohn *et al.*, 2013); however, it should be noted that these studies were performed under static culturing conditions. The work of Jang *et al.* investigated the effects of shear stress on cisplatin nephrotoxicity using primary proximal tubule cells and found significantly different results between static and fluidic culturing conditions (Jang *et al.*, 2013). They noted a lower baseline injury in fluidic cultures and that after cisplatin treatment, cultures grown under a low shear (0.2 dyn/cm²) had a better overall recovery, with a significantly lower degree of apoptosis and higher cell attachment than static comparisons. Here, we investigated the effects of shear on cisplatin and cyclosporine-induced nephrotoxicity on HK-2 cells. Cells were exposed to media that had been treated with cyclosporine, cisplatin, or vehicle control. Cultures were initially exposed to these treatments for 24, 48, and 72 h under static conditions to determine the exposure time necessary to elicit changes in cell viability. It was determined that after 24 h of exposure, cell viability was significantly decreased from controls. To determine whether or not shear stress played a role in cytotoxicity, static and fluidic (0.2 dyn/cm²) cultures were exposed to these pharmaceutical compounds for 24 h and monitored for AP activity, and NGAL and fibronectin secretion. Starting with AP, static and fluidic controls and vehicles were within range of one another, but cyclosporine- and cisplatin-exposed cultures had significantly higher AP in fluidic conditions compared to the static cultures treated in parallel. NGAL secretion was significantly higher in fluidic cultures only (relative to controls), with differences observed in cyclosporine and cisplatin treatments (no vehicle effect was observed). The presence of fibronectin in the cell effluent was increased in cisplatin-treated cultures under fluidic conditions, but no effect was found on static cultures. Additionally, cyclosporine exposure caused a minimal increase in fibronectin secretion (interestingly, a significant decrease in secretion was observed with the EtOH vehicle for cyclosporine in static and fluidic cultures). It was also noted that NGAL and fibronectin secretion was significantly lower in fluidic controls compared against static cultures that were grown in parallel. This might indicate that cells were overall "healthier" under physiological shear conditions, and that they might be more sensitive and responsive to cytotoxicity. These differences in cell responses, specifically the lack of response in static cultures follows along with the observed lack of endocytosis in static cultures that we have found in our past studies with static HK-2 cultures (Sakolish and Mahler, 2017). Primarily, these compounds may not be internalized to the same degree as observed in fluidic cultures. We believe that this fluidic *in vitro* model of the proximal tubule provides a more realistic representation of renal disease and toxicity than static cultures of HK-2 cells, potentially leading to more predictive responses to renal injury.

V. CONCLUSIONS

Here, we investigate the effects of shear stress on disease states caused by increased GFR and hyperglycemia, the effects of renal calculi, and pharmaceutical nephrotoxicity. Many of these conditions have previously been investigated through the use of static cell culturing; however, the effects of a dynamic microenvironment still remain uncharacterized. In this study, it was shown that shear stress can play major roles in these disease conditions, resulting in responses that vary significantly from the static cultures that were run in parallel. It was also shown that shear stress can play significant roles in drug-induced nephrotoxicity, indicating that the lack of fluid shear stress in the *in vitro* cultures used today for pre-clinical drug evaluations significantly reduces the predictive power of these modeling techniques. The use of microphysiological techniques may provide more realistic responses to disease and toxicity, allowing for more physiologically relevant *in vitro* studies on human biological systems.

SUPPLEMENTARY MATERIAL

The [supplementary material](#) contains more detailed protocols on microfluidic device fabrication and assembly, immunocytochemistry, detection of biomarkers, and calcium oxalate crystal synthesis and characterization.

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