Development of a microphysiological model of human kidney proximal tubule function

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The kidney proximal tubule is the primary site in the nephron for excretion of waste products through a combination of active uptake and secretory processes and is also a primary target of drug-induced nephrotoxicity. Here, we describe the development and functional characterization of a 3-dimensional flow-directed human kidney proximal tubule microphysiological system. The system replicates the polarity of the proximal tubule, expresses appropriate marker proteins, exhibits biochemical and synthetic activities, as well as secretory and absorptive processes associated with proximal tubule function in vivo. This microphysiological system can serve as an ideal platform for ex vivo modeling of renal drug clearance and drug-induced nephrotoxicity. Additionally, this novel system can be used for preclinical screening of new chemical compounds prior to initiating human clinical trials.


KEYWORDS: cell polarity; cell survival; proximal tubule

Several publicly funded initiatives now seek to drive the development of innovative, human cell-derived preclinical models that would accelerate the drug development process, shorten the time it takes to move a new molecular entity into early clinical trials, and reduce the excessively high failure rate of clinical trials. Our goal is to apply flow-directed microphysiological technologies to model the physiological functions of the human kidney proximal tubule, as it plays a vital role in active secretory and absorptive transport of drug molecules and is a primary site of drug-induced nephrotoxicity due to these concentrative processes. Whereas existing cell culture and animal models of proximal tubular function have utility, there are serious limitations stemming from functional deficits of conventional cell culture systems and differing physiology between animal models and humans.

In this report, we describe the development of a 3-dimensional (3D) microphysiological system (MPS) of the human proximal tubule. The kidney tubule MPS exhibits long-term viability, retains polarized expression and function of proteins essential for absorptive and secretory transport, responds to physiological stimuli, and performs critical biochemical synthetic activities.

RESULTS

Structural recapitulation of the proximal tubule microenvironment

The stepwise construction of a microphysiological model of human proximal tubule is presented in Figure 1a i to iv. Human primary proximal tubular epithelial cells (PTECs) were grown in monolayer cultures following isolation from renal cortical tissue. After about 7 days in culture, the cell monolayers displayed a uniform cobblestone-like appearance (Figure 1a ii).2,3 Following seeding into the MPS, PTECs adhered to the channel surface and grew to form a tubule-like structure (Figure 1a iv); its physical dimension is close to that reported for the proximal portion of the renal tubule in the human kidney (i.e., 6 mm long × 120 μm thick in the MPS compared with 14 mm long × 40 μm thick in vivo).4 In its present format, the MPS holds approximately 5000 PTECs. Microfluidic technology allowed media perfusion, which

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exposed PTECs to fluid shear force at the apical surface and delivered nutrients continuously under normoxic conditions (Figure 1a).\textsuperscript{5,6} PTEC in 3D culture exhibited excellent viability (>95%) for up to 4 weeks, as demonstrated by extensive green fluorescent calcein acetoxymethyl signal in live cells with minimal red fluorescent signal indicative of

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*Figure 1 | PTEC viability and basic functionality in human kidney 3D MPS. (a) Scheme depicting construction of human proximal tubular epithelial cells (PTECs) in a microphysiological system (MPS). (A1) Cell isolation from human kidney cortex. (A2) Cell culture in 2 dimensions. (A3) Cell seeding and culture in 3-dimensional (3D) MPS. (A4) Phase contrast and viability of PTECs in MPS at day 28. (b) A 3D projection of MPS matrix shows PTEC tubule structure: (B1, 2) surface expression of epithelial cell marker CD13 (red) (original magnification $\times400$); (B3, 4) cell self-assembly confirmed by E-cadherin expression (red) (original magnification $\times400$); (B5, 6) proximal tubule origin confirmed by expression of aquaporin 1 (red) (original magnification $\times400$). (c) Polarization confirmed by (C1) tight junction formation via apical localization of ZO-1 (green) and (C2) basolateral expression of Na\textsuperscript{+}/K\textsuperscript{+} adenosine triphosphatase (green). Tubule diameter is $\sim 120$ μm. Bars = (a(ii)) 200 μM; (a(iv)) 50 μM; (b,c) 20 μM.*
dead cells (Figure 1a–iv). Cell surfaces showed fairly even expression of CD13 (aminopeptidase-N) and E-cadherin, markers of epithelial origin, for at least 28 days (Figure 1b1 to B4). Proximal tubule origin of the final culture was verified by consistent immunohistochemistry staining for aquaporin-1 (Figure 1b5 and B6) and lotus lectin (Supplementary Figure S1), whereas the signal for aquaporin-2, prominin 2, and uromodulin, markers of distal tubule or collecting duct cells or both, was absent in both 2-dimensional (2D) cultures and MPS (Supplementary Figure S1). It is interesting to note that KIM-1, a marker of acute kidney injury, is expressed in PTECs cultured in 2D, but is absent in MPS devices (Supplementary Figure S1). This suggests that flow-directed culture coupled with 3D architecture confers a less injured, more quiescent phenotype within the MPS.

Transmission electron microscopy images of PTEC ultrastructure showed representative density of mitochondria, Golgi apparatus, and rough endoplasmic reticulum. Tight junctions and short microvilli at the apical surface were also observed, as well as basolateral interdigitations between neighboring cells, the latter being characteristic of proximal tubules in vivo (Figure 2a1 and 2 and Supplementary Figure S2).7 Polarization of PTECs was shown by localization of the tight junction protein ZO-1 to the luminal (apical) aspect of the PTEC tubule, and localization of Na+/K+-adenosine triphosphatase (ATPase) to the lateral interface between neighboring cells and the basal border between PTECs and collagen substrate (Figure 1c). Cilia formation in response to fluid shear stress was evidenced by positive staining of acetylated tubulin in rod-like structures that originate close to the cell nucleus; the ciliary process averaged 10 ± 3.5 µm in length (Figure 2b1 and 2).

**Recapitulation of proximal tubule physiological functions**

Reclamation of glutathione is an essential biochemical function of renal proximal tubule in vivo.8 The reclamation process is mediated by γ-glutamyl transpeptidase (GGT), which normally mediates the transfer of glutamyl moiety from glutathione to an acceptor amino acid as part of the γ-glutamyl cycle, a pathway for the synthesis and degradation of glutathione. Selective immunohistochemistry staining for GGT in the MPS revealed enriched localization of the enzyme at the luminal aspect of the PTEC structure (Figure 3a). GGT mediates a comparable reaction for the oxidized form of glutathione, glutathione disulfide (GSSG) (Figure 3b), which is a more chemically stable substrate for assessing the activity of γ-glutamyl transpeptidase in proximal tubule MPS. PTECs within the MPS were perfused with media containing 4 µM GSSG in the presence or absence of the irreversible GGT inhibitor acivicin (1 mM). The recovery of GSSG in the effluent was low (<1.5%), demonstrating extensive catalytic activity of GGT; in the presence of acivicin, an approximate 2-fold increase in GSSG recovery was observed over 2 to 4 hours (Figure 3c).

The proximal tubule is responsible for nearly 90% of glucose reabsorption in the kidney, which is mediated primarily by glucose transporters, including SGLT2.10 Selective immunohistochemistry staining for SGLT2 in the proximal tubule revealed its expression and localization at the apical surface (Figure 4a). To confirm functioning of SGLT2 in the PTEC tubule microenvironment, glucose reabsorption was demonstrated by perfusing the proximal tubule in the MPS with a fluorescent glucose analog, 2-NBDG (200 µg/ml) in the presence or absence of either a SGLT2/glucose transporter inhibitor (apigenin 50 µM) or a SGLT2-specific inhibitor.

**Figure 2 | Ultrastructure of human PTECs in human kidney 3D MPS.** (a1, 2) Transmission electron microscopy depicting ultrastructure of proximal tubular epithelial cells (PTECs) cultured in a microphysiological system (MPS) device. The following cellular structures are identified: microvilli (MV), mitochondria (M), tight junction (TJ), endoplasmic reticulum (ER), and Golgi apparatus (GA). Original magnifications (a1) × 10,000 and (a2) × 30,000. (b1, 2) PTECs in MPS form cilia as seen from 2 representative images of single cells stained for acetylated tubulin in red. Bars = (a1, 2) 500 nM and (b1) 5 µM). 3D, 3-dimensional.
dapagliflozin 0.5 μM). PTEC layer showed strong green fluorescent signal demonstrating avid cellular uptake and accumulation of 2-NBDG. Fluorescent signal intensity of 2-NBDG decreased significantly in the presence of both inhibitors, indicating blockade in uptake of the fluorescent glucose analog into epithelial cells (Figure 4b to f). It is worth noting that (Figure 3 | GGT activity in human kidney 3D MPS. (a) Immunocytochemistry reveals proper apical localization of γ-glutamyl transpeptidase (GGT) (green) in juxtaposition to nuclei (blue) within the proximal tubular epithelial cell tubule. (b) GGT is functionally essential to cleaving the γ-glutamyl moiety from oxidized glutathione and can be inhibited by acivicin. (c) GGT activity as determined by oxidized glutathione abundance in the presence and absence of inhibitor, acivicin (n = 4 microphysiological system [MPS] devices) *P < 0.001, 2-tailed t-test. Bars = 20 μM for the tubule and 10 μM for the wall. 3D, 3-dimensional; GSSG, glutathione disulfide.}

Figure 4 | Glucose reabsorption in human kidney 3D MPS. (a) Glucose is actively reabsorbed from the urine via sodium glucose cotransporter-2 (SGLT2) located on the apical membrane in the proximal tubular epithelial cell tubule. Immunocytochemistry reveals proper apical localization of SGLT2 (green) in juxtaposition to nuclei (blue). (b,d) Differential interference contrast images showing the structure of proximal tubular epithelial cells in the microphysiological system (MPS) in the presence and absence of SGLT2 inhibitor, apigenin. (c,e) Fluorescent images showing the distribution of the fluorescent glucose analog, NBDG. (c) NBDG, a fluorescent glucose analog, was actively reabsorbed in the absence of inhibitor and (e) was not absorbed in the presence of inhibitor. (f) Quantification of a cell-associated fluorescent signal following subtraction of autofluorescence, demonstrating significant reduction of glucose uptake in the presence of inhibitors apigenin and dapagliflozin (n = 3 MPS devices/group) *P < 0.001, unpaired t-test. Bars = (a) 20 μM for the tubule and 10 μM for the wall and (e) 50 μM. 3D, 3-dimensional.
MPS responded with an approximate 3-fold increase in luminal perfusate pH from 7.4 to 6.9. PTECs in the physiological response, PTECs were exposed to a decrease in secretion of ammonia (Figure 6a). To demonstrate this, blood or luminal triphosphate; DMSO, dimethylsulfoxide.


Figure 5 | Cellular ATP content in control and antimycin A-treated (1 μM, 24 hours) MPS. (n = 3 microphysiological system [MPS] devices/groups). *P = 0.05, 2-tailed t-test. ATP, adenosine triphosphate; DMSO, dimethylsulfoxide.

noting that the magnitude of effect for both inhibitors (25%–30%) is similar to the clinical efficacy of SGLT2 inhibitors (30%–50%) despite in vitro predictions of 90% inhibition.11

To demonstrate that the primary cells we use to populate the MPS generate their ATP energy source from mitochondrial oxidative phosphorylation as opposed to aerobic glycolysis (i.e., the Warburg effect),12 we measured cellular ATP levels in the presence and absence of antimycin A. As seen in Figure 5, exposure to 1 μM antimycin A for 24 hours resulted in a 2-fold drop in ATP content. A similar magnitude decrease was also observed in PTECs cultured in monolayer (data not shown).

Renal proximal tubules respond in vivo to a drop in either blood or luminal filtrate pH with an increased generation and secretion of ammonia (Figure 6a). To demonstrate this physiological response, PTECs were exposed to a decrease in MPS luminal perfusate pH from 7.4 to 6.9. PTECs in the MPS responded with an approximate 3-fold increase in effluent ammonia concentration (pH 7.4–0.55 mM NH3, pH 6.9–1.56 mM NH3) (Figure 6b).

The proximal tubule epithelium is known to be a critical site for bioactivation of vitamin D. Systemically available 25-OH vitamin D₃ (calcidiol) is converted to bioactive 1α,25-(OH)₂ vitamin D₃ (calcitriol) through the action of cytochrome P450 27B1 (CYP27B1). Metabolism of calcidiol to relatively inactive metabolites also occurs in the kidney via cytochrome P450 24A1 (CYP24A1). When seeded into the MPS, PTECs demonstrated the ability to metabolize calcidiol to quantifiable levels of bioactive calcitriol, inactive 24,25-(OH)₂ vitamin D₃ and 4β,25-(OH)₂ vitamin D₃ (Figure 7a).13 Overall, the formation clearance (i.e., formation rate normalized by substrate concentration) for 24,25-(OH)₂ vitamin D₃ was significantly greater than that of calcitriol and 4β,25-(OH)₂ vitamin D₃. This finding is consistent with metabolism of calcidiol in vivo, where circulating concentrations of 24,25-(OH)₂ vitamin D₃ are known to be higher than those of both calcitriol and 4β,25-(OH)₂ vitamin D₃.14 In order to further explore vitamin D homeostasis, MPS-seeded PTECs were challenged with additional exogenous calcitriol (500 nM), a vitamin D receptor ligand. Induction of the vitamin D receptor–regulated 24-hydroxylation pathway was observed (Figure 7b). This increase in 24,25-(OH)₂ vitamin D₃ formation was accompanied by a rapid (5 hours) and sustained (72 hours) accumulation of mRNA transcripts for CYP24A1 (Figure 7c). No apparent changes in gene expression of CYP27B1 (Figure 7d) or vitamin D receptor (Figure 7e) were observed.

**Secretory transport of organic solutes**

Circulating organic solutes, including drugs and xenobiotics, can undergo secretion into the tubular lumen by means of active transport across the renal tubule epithelium. Secretory transport of the prototypical organic anion, para-aminohippurate (PAH) across PTEC monolayers was evaluated in both conventional Transwell (Corning Corp., Corning, NY) permeable cell culture inserts and the MPS, in the presence and absence of a competitive inhibitor, probenecid. No change in apparent permeability was observed in the 2D Transwell system upon addition of probenecid (Figure 8a). However, within the MPS, the relative appearance of PAH in the effluent from the PTECs cultured in monolayer decreased approximately 4-fold in the presence of probenecid (Figure 8b). These data support the involvement of basolateral uptake (OAT1/3) or apical efflux (MRP2/4) transporters or both in the observed vectorial transport of PAH in the MPS.15

We further evaluated transport in the MPS using the endogenous anionic uremic solute, indoxyl sulfate (Figure 9a). As compared to PAH, indoxyl sulfate output in the PTEC channel effluent was approximately 20% lower, but exhibited a similar degree of inhibition by probenecid. In addition, an interaction between PAH and indoxyl sulfate was demonstrated; coperefusion with indoxyl sulfate decreased secretory transport of PAH by 1.8-fold, suggesting a competition of the 2 solutes for the same organic anion transporters (Figure 9b).

**DISCUSSION**

We have demonstrated that in a flow-directed MPS, human PTECs attach to supportive collagen extracellular matrix (ECM) and self-assemble to form a 3D tubular structure.5,6 Furthermore, cells maintain renal epithelial differentiation and characteristic morphology in this microenvironment for an extended period of time. In contrast, PTECs grown in conventional 2D monolayer cultures often show limited longevity and loss of distinctive epithelial phenotype due to lack of fluidic mechanosensory input16 and other stimulus elements present in the native microenvironment in vivo. Also, in contrast to conventional 2D culture, PTECs grown in the MPS polarize with proteins selectively localized to the basolateral and apical aspects of the tubular epithelium and exhibit expected morphological and functional phenotypes of proximal tubule epithelium in vivo out to 28+ days.7,17

We also demonstrated proximal tubular origin for the majority of epithelial cells in culture by immunohistochemistry staining of differential markers of kidney epithelial cells.
**Figure 6 | Ammoniagenesis in human kidney 3D MPS.** (a) The physiological response to a drop in either blood or luminal pH resulting in the generation and secretion of ammonia in the tubular outflow. (b) With the microphysiological system (MPS), luminal media was initially at pH 7.4 and then switched to pH 6.9. Secreted ammonia in the outflow was quantified spectrophotometrically from 4 separate devices after 4 hours and was significantly different when exposed to acidic conditions. *P = 0.05, 2-tailed t-test. 3D, 3-dimensional; α-KG, alpha ketoglutarate; GLN, glutamine; GLU, glutamic acid.

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**Figure 7 | VDR-mediated regulation of vitamin D metabolism in human kidney 3D MP.** (a) Formation clearance of the 1α,25-(OH)2-vitamin (Vit) D3 (calcitriol), 4β,25-(OH)2-Vit D3, and 24,25-(OH)2-Vit D3 over 3 days exposure of proximal tubular epithelial cells (PTECs) cultured in microphysiological system (MPS) to 1 μM 25-OH-Vit D3 (calcidiol). Clearance values are plotted at midpoint of collection interval. Sequential metabolism was assumed to be negligible. (b) Formation clearance of the 24,25-(OH)2-Vit D3 was greater in MPS-seeded PTEC cultured in media with both 0.5 μM 1α,25-(OH)2-Vit D3 (calcitriol) and 1 μM 25-OH-Vit D3 (calcidiol) than those exposed to 1 μM calcidiol alone. Clearance values were plotted at the midpoint of the collection interval. Sequential metabolism was assumed to be negligible. Baseline data point (×) for formation clearance (CLf) was determined from the experiment presented in (a). Effect of calcitriol on gene expression of (c) CYP24A1, (d) CYP27B1, and (e) vitamin D receptor (VDR) in MPS-seeded PTEC. Relative accumulation of CYP24A1 mRNA transcripts was greater in MPS-seeded PTEC cultured in media with both 1α,25-(OH)2-Vit D3 (calcitriol) and 25-OH-Vit D3 (calcidiol) than those exposed to calcidiol alone. Induction of CYP24A1 mRNA occurred rapidly (5 hours) and persisted for the duration of treatment (3 days). No detectable (ND) transcripts of CYP24A1 were observed in the 5-hour calcidiol-only group. There were no substantial changes in CYP27B1 and VDR mRNA expression over the duration of treatment. All genes of interest were standardized to glyceraldehyde-3-phosphate dehydrogenase. CYP, cytochrome P450.
(viz., aquaporin 1, aquaporin 2, and SGLT2). Structurally, PTECs in the MPS exhibited polarized structure based on localization of domain-marker proteins. Assessment of ultrastructure by transmission electron microscopy revealed the hallmarks of a competent epithelial barrier, as well as healthy mitochondria and characteristic basolateral membrane interdigitations. The apical brush border expressed functionally active SGLT2 transporter (Figure 4) and GGT enzyme (Figure 3). Although brush border microvilli on the cultured PTECs were not as abundant as in freshly isolated cells,6,17 this is consistent with literature reports of low microvilli density in traditional PTEC monolayer cultures.2,18 Metabolic competence of the cultured PTECs was confirmed by their capability for ammoniagenesis and vitamin D biotransformation. In the case of vitamin D, this metabolic competence was accompanied by retention of some of the machinery critical to maintaining vitamin D homeostasis in vivo.

The human kidney tubule MPS recapitulates the perfusion delivery and transport pathway of a secreted solute in vivo in that the test solute is perfused into a surrogate vascular channel, diffuses through the pseudo-interstitial space, and undergoes uptake and efflux across the epithelial barrier into the flowing perfusate in the tubular luminal channel. We demonstrated robust transepithelial transport of organic anionic (PAH, indoxyl sulfate) solutes that were sensitive to selective transport inhibitors. The results showed some inter-MPS and intra-MPS variability, which can be attributed to (i) interindividua variability in expression of proximal tubule transporters, and (ii) instability in effluent flow as a result of the porous and pliable collagen ECM. Nonetheless, the human proximal tubule MPS demonstrates applicability for assessing renal tubular secretion of drugs that was not observable using PTECs in a Transwell system. Our work lays a foundation for further investigation of the complex, coordinated uptake and efflux transport processes at the tubular epithelium.

A number of 3D tissue engineering models have been developed that attempt to mimic the proximal tubule structure and function8,16,19,20 (for reviews see Kelly et al.5 and Kim and Takayama21). A number of these systems utilize a monolayer of cells adhered to a microporous membrane coated with a thin layer of an ECM protein component, often collagen IV or laminin. The importance of 3D tubular architecture is reinforced by the observation that transplantation of 2D sheets of porcine renal proximal tubule cells onto dorsal subcutaneous tissue of nude rats results in increasingly complicated tubular structures with altered expression of surface markers as the 2D sheets convert to their native structure in vivo.22 It has become increasingly evident that the composition,23 microtopography,24,25 and rigidity/elasticity26,27 of the ECM substrate on which a cell adheres can dictate the cells’ morphology, phenotype, proliferation, and even fate. Unlike systems using microporous membranes,
the tubular structures formed in our microphysiological system are surrounded by ECM whose composition and rigidity/elasticity can be modified to best recapitulate in vivo-like cellular function. Additionally, our microfluidic system enables coculture of a variety of cell types in the matrix compartment and the proximal tubule channel, permitting critical cell-cell and cell-matrix interactions, without interference from artificial scaffold materials.

The system described herein represents a milestone in cell culture modeling of human kidney in that it is possible to achieve a functional 3D construct of human proximal tubule in vitro in a microfluidic device that more faithfully reflects the native microenvironment of the renal tubulo-interstitium. There have been earlier attempts in recapitulating a functioning 3D proximal tubule. DesRochers et al. constructed a static 3D culture of proximal tubular cells by culturing immortalized human renal cortical epithelial cells (NKi-2) suspended in an ECM consisting of a 50:50 mixture of rat tail collagen I and Matrigel (BD Bioscience, Billerica, MA). Maschmeyer et al. cultured immortalized cell line renal proximal tubular epithelial cell/telomerase reverse transcriptase 1 (RPTEC/TERT-1) on a polyester track etched membrane within a 4-organ–chip device under continuous flow. Although longevity in culture and reproducible sourcing are practical advantages, the altered biology of immortalized cell lines is a serious drawback. The observation that the PTECs in our MPS self-assemble into 3D tubular structure may help to recapitulate proximal tubular functionality more effectively than previous monolayer or dispersed culture systems have done.

More recently, Jang et al. reported development of a “kidney on a chip” with similar goals to our project. The MPS reported by Jang et al. consisted of a PTEC epithelial monolayer exposed to an apical fluid shear stress that was capable of reabsorbing glucose in the range of approximately 2% of the nominal input. Notably, our kidney tubule MPS achieves approximately 10-fold higher glucose reabsorption than that noted by Jang et al., which is responsive to SGLT-2 specific inhibition and thus more closely replicates expected physiological functions. Efficient apical glucose uptake is among the key functions of proximal tubules in vivo. In addition, our proximal tubule MPS also exhibits organic solute transport functions that will permit its application in the study of renal drug clearance mechanisms. In contrast to the work of Jang et al., we are also able to demonstrate physiologically regulated PTEC CYP-450 function in the biosynthesis of vitamin D sterols. Finally, our system demonstrates maintenance in vitro of an epithelial cell phenotype for up to 28 days, whereas the system employed by Jang et al. was tested for only up to 4 days.

In pioneering work by Humes et al., the development of a renal tubule assist device (RAD) also recapitulated multiple aspects of proximal tubular physiological function ex vivo. Similar to what we observe in our MPS, the RAD also demonstrated glucose reabsorption, glutathione metabolism, and PAH secretion. The RAD, as the name implies, was developed for the purpose of providing proximal tubule replacement therapy in the setting of kidney failure, whereas our kidney on a chip was designed for the specific purpose of improving the drug development as part of the National Institutes of Health Organs on Chips Consortium. Thus the RAD was designed to support renal function in vivo on a macroscale, whereas our MPS is primarily designed for predictive toxicity testing on a microscale. Given the divergent goals for development of the RAD and our proximal tubule on a chip, it is not surprising that each system has both comparative strengths and weaknesses. In the RAD system, media is perfused across a flat cell sheet in a 1-mm wide channel that attempts to mimic luminal shear stress. In contrast, our microphysiological system enables the creation of a 120-μm diameter tubular tissue that is in direct contact with the surrounding 3D ECM, which more closely replicates human anatomy. Additionally species specificity for individual transporters may be a concern, given the use of primary porcine cells in the RAD device, although the RAD tested in clinical trials did make use of human cells. Because of the thickness of the hollow fiber dialysis membrane, the RAD may be less efficient for assessing basolateral secretory organic solute clearance, which is the major mechanism for proximal tubule drug and metabolite elimination.

Conversely, because the hollow fiber design of the RAD allows facile abluminal flow and creation of an interstitial oncotic gradient favoring apical reabsorption, the RAD is superior to the current kidney on a chip design for detecting apical water and electrolyte reabsorption. Despite attempts to measure ion flux in our MPS using lithium as a marker of sodium transport in the presence and absence of inhibitors targeting either Na+/H+ exchanger or Na+/K+ ATPase, we were unable to recapitulate this function (data not shown). Although this is not a major limitation of the MPS for assessing organic drug elimination by the kidney as there is limited active apical reabsorption of organic xenobiotics, nonetheless we are currently developing a 2-channel microfluidic kidney MPS, with the second channel allowing a peritubular microvascular endothelia to be grown within the matrix compartment. This design should provide a biomimetic for a more complex in vivo-like microenvironment that may facilitate assessment of electrolyte and water reabsorption in the proximal tubule. In addition, several studies have shown that coculturing endothelial cells with proximal tubule epithelial cells enhances in vivo-like epithelial function.

In summary, for the first time, we have a high-fidelity system in vitro that allows reliable investigation of the fundamental biology of the renal tubule epithelium; the potential applications include tubular secretion of drugs, xenobiotics, and uremic toxins, as well as the ability to assess toxic injury response. To our knowledge, this is the first demonstration of a human proximal tubular cell in vitro system that can effectively model basolateral solute transport, apical solute uptake, and intracellular enzymatic function in a physiologically relevant manner. Moreover, our human kidney
proximal tubule microphysiological system has the potential for integration of multiple cell types—epithelial cells, pericytes, and microvascular endothelial cells—in a spatial alignment that would fully reconstitute a tubulointerstitial environment. In time, we envision the MPS will be extended to investigation into pathophysiological processes at the tubule-interstitium that underlie acute kidney injury and chronic kidney diseases, as well as transport and metabolic processes governing the renal handling of drugs and environmental toxicants in healthy and disease states.

MATERIALS AND METHODS
Cell isolation, culture, and seeding in MPS platform
Human kidney tissue was obtained from specimens obtained during surgical resection of renal cell carcinoma performed at the University of Washington Medical Center. The human subject protocol was approved by the University of Washington Institutional Review Board. Healthy portions of the surgical specimen were dissected, stored at 4 °C in Hanks balanced salt solution buffer containing penicillin-streptomycin, and processed for isolation of proximal tubule epithelial cells within 24 hours as described previously. Cells with passage numbers 1 through 4 were used in the subsequent 3D culture experiments—please refer to Supplementary Figure S4 for consistency of morphological appearance of cells across donors and passage numbers.

The 3D MPS platform used in these studies was developed by Nortis Inc. (Seattle, WA) and details of its construction have been reported by Tourovskaia et al. For cell seeding, MPS platforms were filled with ECM of 6 mg/ml rat tail collagen type I (Ibidi, Madison, WI) at 4 °C. MPS platforms were left for 30 minutes at 4 °C for matrix settling, and then stored at room temperature overnight. Microfibers inserts were removed from the device and the channel was coated with collagen type IV (5 µg/ml; BD Biosciences, Bedford, MA) over 1 hour.

Confluent monolayer cultures of PTECs were treated with 0.05% Trypsin-ethylenediamine tetraacetic acid to obtain single-cell suspensions. PTECs were washed, counted, and resuspended at a concentration of 15 to 20 × 10^5 cells/ml and ~5 µl of cell suspension was injected into the collagen IV–coated lumen of the MPS. Cells were allowed to adhere for 24 hours before initiating media flow at 0.5 µl/min. Cell coverage and integrity of the tubule structure were assessed under light microscopy on a weekly basis. Additionally, cell viability was assessed using a LIVE/DEAD Viability/Cytotoxicity assay (Invitrogen, Carlsbad, CA). For all of the experiments conducted, MPS platforms contained PTECs grown for 2 to 3 weeks after initial cell seeding.

Immunocytochemistry and imaging
Proximal tubule epithelial cells in MPS platforms were fixed with 10% Buffered Formalin Acetate (Fisher Scientific, Pittsburg, PA) for 1 hour, permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO), and blocked with 0.1% bovine serum albumin for 40 minutes. Primary antibodies were then applied overnight at 4 °C and fluorescently labeled secondary antibodies were applied for 1 hour at room temperature. Cells were mounted using SlowFade gold (Invitrogen, Carlsbad, CA) medium. Phase contrast and fluorescent images were acquired as described in Supplementary Materials and Methods. For analysis of MPS ultrastructure by transmission electron microscopy, devices were fixed in 1/2× Karnovsky fixative (2.5% glutaraldehyde with 2% formaldehyde in 0.1 M buffer). Samples were postfixed in osmium tetroxide and processed, sectioned, and examined according to protocols described previously.

GGT activity
The presence and functioning of GGT was demonstrated by metabolism of GSSG or the oxidized form of glutathione in MPS cultured with PTECs grown to >50% confluence. To test for GGT activity, proximal tubule MPSs were perfused with culture media at 1 µl/min containing 4 µM of GSSG; effluent samples were collected at hourly intervals for a total of 4 hours. After the first phase of collection, the platforms were perfused overnight using fresh media to ensure sufficient washout of GSSG. To verify specificity of GSSG processing by GGT, platforms were reperfused with 4 µM GSSG in the presence of GGT inhibitor–acivicin (1 µM), and effluent samples were collected hourly for a total of 4 hours. GSSG concentration in the effluent perfusate was measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Results are presented as recovery of GSSG expressed as a percentage of nominal input in the presence and absence of GGT inhibitor. Additional analytical details are presented in Supplementary Materials and Methods.

ATP assay
Generation of ATP via cellular respiration by PTECs was tested. MPS-cultured PTECs were treated with 1 µM antimycin A (Abcam, Cambridge, MA) or 0.01% dimethylsulfoxide for 24 hours followed by measurement of ATP using a luminescent ATP detection kit (Abcam) with slight modification. Briefly, cells were removed from the devices by injecting approximately 50 µl of detergent (Abcam luminescent kit, part 8206000) into the injection port allowing cells to be collected at the luminal outflow port. Cell lysates were transferred to a white opaque 96-well plate (Corning Costar, Corning, NY) to complete the kit procedure and luminescence was quantified using a PlateLumino luminometer (Stratec Biomedical Systems AG, Geverbest, Germany). A 5-point ATP standard curve (10 µM–1 mM) was generated and used to convert relative luminescence units to ATP concentration.

Ammoniagenesis
The ability to secrete ammonia in response to acidification was evaluated in MPS platforms cultured with PTECs grown to >50% confluence. The MPSs were initially perfused with phosphate-buffered saline (pH 7.4) at a flow rate of 1 µl/min for 4 hours; effluent was collected at 2-hour intervals. After 4 hours, the perfusate was switched to phosphate-buffered saline buffered to an acidic pH of 6.9, and 2-hourly collection of effluent continued for another 4 hours. Samples at hour 4 were analyzed for ammonia concentration using a colorimetric ammonia assay kit (Abcam). Effluent ammonia concentrations at acidic and physiological pH conditions were compared.

Glucose reabsorption
Glucose reabsorption via SGLT2 was demonstrated in the MPS. Platforms were perfused with media for at least 2 weeks prior to being tested for glucose reabsorption. Glucose uptake was assessed using a fluorescent glucose analog 2-NBDG (2-deoxy-2-[7-nitro-2, 1, 3-benzoxadiazol-4-yl] amino)-D-glucose; Cayman Chemicals, Ann Arbor, MI). Twelve hours prior to testing, PTEC perfusion was switched to culture media with a lower, physiological glucose concentration (100 mg/dl). After 12 hours of pretreatment, MPS platforms were perfused with media containing 200 µg/ml (0.6 mM) of 2-NBDG either in the absence or presence of inhibitors apigenin
(50 μM) or dapagliflozin (0.5 μM) for 2 hours at 37 °C. Platforms were then washed and imaged using a Nikon Eclipse Ti-S (Tokyo, Japan) and inverted spinning disk microscope in phase contrast and fluorescent modes and fluorescent signal quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

25-(OH)₂ vitamin D₃ metabolism
Conversion of 25-OH vitamin D₃ to its physiologically active and inactive metabolites was assessed in the MPS. Cells cultured from 3 separate donors were evaluated in triplicate for a total of 9 MPS platforms. PTECs were cultured for 2 to 3 weeks prior to the addition of 1 μM 25-OH vitamin D₃ (Toronto Research Chemicals, Toronto, Canada) to the cell culture media containing 2% fetal bovine serum to minimize adsorption of vitamin D₃ metabolites. Inflow rate of 25-OH vitamin D₃-containing media was set at 0.5 μl/min and outflow was collected daily for 3 days and stored at –80 °C. LC-MS/MS analysis of 25-OH vitamin D₃ and its primary metabolites was conducted using a previously established method with modifications described in the Supplementary Materials and Methods. Formation clearance for the 3 dihydroxy-vitamin D₃ metabolites (12,25-(OH)₂ vitamin D₃, 1,25-(OH)₂ vitamin D₃, and 24,25-(OH)₂ vitamin D₃) was plotted over time. The method for calculating the formation clearance is described in Supplementary Materials and Methods.

Vitamin D receptor-mediated regulation
To evaluate the inductive effects of 1α,25-(OH)₂ vitamin D₃ (calcitriol) on the 24-hydroxylation of 25-OH vitamin D₃ (calcidiol), a group of MPS devices (n = 5) were exposed to 1 μM calcidiol in the presence or absence of 0.5 μM exogenous calcitriol. Effluent was collected for 3 days and stored at –80 °C until LC-MS/MS analysis of 24,25-(OH)₂ vitamin D₃ and calcidiol concentrations. Following treatment, RNA was extracted using Tri-reagent. In order to assess the more rapid effects of calcitriol coadministration on gene expression, additional MPSs were treated for 5 hours prior to collection in Tri-reagent. Total RNA was reverse transcribed to cDNA using reverse transcription kit (Life Technologies, Carlsbad, CA). Assessment of the effects of calcitriol on the expression of genes relevant to calcidiol metabolism (CYP24A1, CYP27B1, and vitamin D receptor) was conducted using TaqMan Gene expression assays (Life Technologies) on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Relative quantification of mRNA was determined using glyceraldehyde-3-phosphate dehydrogenase as the housekeeping gene.

Secretory transport of organic solutes
Secretory transport of solutes was assessed using dual-channel MPS platforms as depicted in Supplementary Figure S3; 1 channel was populated with PTECs with an average of 85% confluent cell coverage, the other channel remained cell-free and served as a pseudo-vascular channel for delivery of substrates to the basolateral aspect of the tubular epithelium. Substrate solute was added to the media flowing through the cell-free channel. Competitive inhibitors were introduced into the media flowing through both channels. The rate of flow through both channels was set at 1 μl/min. Effluent from both channels was collected hourly. To assess secretory transport of PAH, 1 μCi/ml 14C-PAH or 2 μM equivalent of PAH or 10 μM indoxyl sulfate (Sigma-Aldrich, St. Louis, MO) was introduced into the cell-free channel. Appearance of solute in the PTEC channel effluent was measured in the presence or absence of 2 mM probenecid. Interaction in secretory transport between PAH and indoxyl sulfate was assessed by examining 1 μCi/ml 14C-PAH transport in the presence of 2 mM indoxyl sulfate. The appearance of radio-labeled PAH in the effluent was measured by liquid scintillation counting. Concentration of indoxyl sulfate in the effluent was measured by a previously established LC-MS assay with modifications. Details on the LC-MS assay are presented in Supplementary Materials and Methods. The MPS experiments depicted in Figures 8b, 9a, and 9b used 9, 8, and 7 MPSs, respectively. A minimum of 3 MPSs were assigned to each inhibitor treatment and control group. Hourly time points were aggregated and presented as a mean ratio of the amount of test substrate appearing in the PTEC channel effluent relative to its nominal input amount into basolateral channel ± inhibitor. Error bars reflect the SEM of the aggregated time points for each experiment. For comparative purposes, transport of PAH was measured in Transwell as described elsewhere. Details on the protocol and modifications are available in Supplementary Materials and Methods.

Statistical analysis
Data are reported as means ± SE. For comparison of means, statistical tests were applied using GraphPad software (La Jolla, CA).

DISCLOSURE
All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL
Supplementary Materials and Methods.
Figure S1. Characterization of renal tubular cell phenotype by immunocytochemistry. (A) Quantification of 4 fields of view for
percentage of cells expressing antigens shown in B to I. (B–I) Representative immunofluorescence images of 2-dimensional cultured renal tubular cells evaluated for (B) sodium glucose cotransporter-2 (SGLT2), (C) aquaporin-1 (AQP1), (D) CD13, (E) aquaporin-2 (AQP2), (F) lotus-lectin, (G) kidney injury molecule-1 (KIM-1), (H) uromodulin, and (I) prominin-2 (PROM2). (J–M) Immunofluorescence images of 3-dimensional microphysiological system—cultured renal cells assessed for (J) lotus-lectin, (K) KIM-1, (L) uromodulin, and (M) PROM2. Cells isolated from renal cortical tissue show considerable staining for proximal tubule antigens (AQP1, lotus-lectin, and SGLT2), whereas minimal staining is observed for antigens expressed in other segments of the nephron (AQP2, uromodulin, and PROM2). Bars = 100 μM.

Figure S2. Demonstration of basolateral intercellular interdigitations (*) in a human kidney 3-dimensional microphysiological system (original magnification ×12,000).

Figure S3. Experimental design for evaluating secretory transport by proximal tubular epithelial cells (PTECs) in a human kidney 3-dimensional microphysiological system. Substrate ± inhibitor is perfused through the pseudo-vascular channel. Inhibitor is also perfused through the receiving PTEC channel. The effluent media is collected from both channels at designated time points.

Figure S4. Cell morphology across donors and passages. (A) Passage 0 cells after 8 days in culture from 68-year-old woman; (B) passage 0 cells after 8 days in culture from 60-year-old man; (C) passage 0 cells after 25 days in culture from 66-year-old man; (D, E) passage 1 cells after 7 days in culture from 63-year-old man; (F) passage 1 cells after 5 days in culture from 70-year-old man; (G) passage 2 cells after 8 days in culture from 59-year-old man; (H) passage 3 cells after 6 days in culture from 71-year-old man; (I) passage 3 cells after 15 days in culture from 81-year-old man. Bars = 100 μM. Supplementary material is linked to the online version of the paper at www.kidney-international.org.

REFERENCES