Development of an *in vitro* 3D Human Kidney Proximal Tubular Epithelial Tissue Model

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Abstract

The renal proximal tubular (PT) region is the most common site for a compound-specific kidney injury. The PT region is responsible for essential kidney functions, including reabsorption of low molecular weight proteins, solutes, and glucose, secretion of acids, and clearance of administered medications. The ultimate goal of this project is to develop a novel physiologically relevant primary human kidney-cell-based 3-dimensional (3D) organotypic tissue model for the prediction of human nephrotoxicity.

Human primary PT epithelial cells (PTEC) were isolated and expanded in a monolayer culture prior to seeding onto microporous membrane inserts to reconstruct a 3D organotypic model. 3D tissues were analyzed by histology, barrier integrity (transepithelial electrical resistance, TEER), immunostaining, and qPCR on days 5 to 30. Receptor-mediated FITC-albumin uptake, glucose uptake, and transpeptidase hydrolytic activity of glutamyl transpeptidase (GGT1) and leucine aminopeptidase (LAP) were assayed on days 10 to 28.

The PTEC organotypic tissues (EpiKidney) organize into characteristic tubular structures, develop a barrier with TEER 110.2+/-33.3 Ω·cm^2 on day 9 and stain positive for tight junction proteins ZO-1, claudin-1, and occludin. The organotypic tissues differentiate into polarized epithelium expressing brush border proteins megalin, villin, and GGT1 together with water channel AQP1 on the apical side and sodium-potassium ATPase pump on the basolateral side. Real-time qPCR analysis confirmed that tissues express a panel of PTEC-specific markers that are necessary for renal clearance, secretion, and reabsorption: aminopeptidase CD13, multidrug resistance proteins MRP2/4, CYP450 enzymes, glucose transporters SGLT1/2, multidrug and toxin extrusion transporter MATE1, organic cation and anion transporters OCT1/2, OCTN1/2, and OATP4C1, urate transporter URAT1, and sodium phosphate co-transporter NP2. Specific concentration-dependent and time-dependent receptor-mediated uptake of FITC-albumin by PTEC tissues was observed by fluorescent microscopy. Significant conversion of γ-Glutamyl-p-nitroanilide (GPNA, 2.5 mM) and L-leucine-p-nitroanilide (LLNA, 3 mM), substrates for GGT1 and LAP respectively, was detected via spectrophotometric monitoring of p-nitroaniline (PNA) following 30 min incubation. Specific transpeptidase hydrolytic activity was inhibited in the presence of an irreversible inhibitor acivicin (1.2 mM) by 88.8% (GGT1) and 35.0% (LAP).

The reconstructed in vitro 3D Kidney-PT organotypic tissue morphology, barrier properties, gene expression, and tissue performance resemble the in vivo human PT region. This model is anticipated to be a valuable tool to evaluate human nephrotoxicity and its mechanisms, improve the predictivity of human responses to pharmacological substances, and help establish confidence in drug development and testing.

Methods

FITC-Albumin Uptake: EpiKidney tissues were pre-incubated in DMEM/F12 medium for 2h followed by application of 300 µg/mL of FITC-Albumin conjugate (#A9771, Sigma) in the presence or absence of 10 mg/mL of BSA for 2h. FITC uptake was analyzed by confocal microscopy of the 3D tissues or the fluorescence spectroscopy (excitation/emission 488/516 nm) of Tx-100 lysed tissues.

Glucose Uptake: EpiKidney tissues were pre-incubated in a glucose-free buffer for 3h followed by 1h incubation in the presence of 1mM of 2-deoxyglucose (2-DG) in a buffer with or without of NaCl (137mM). 2-DG uptake was analyzed by a chromogenic assay following manufacturer's instructions by absorbance at 420nm (#CSR-OKP-PMG-K01TE, Cosmo Bio Co Ltd, Tokyo, Japan).

Hydrolase Activity: L-γ-Glutamyl-p-nitroanilide (GPNA) and L-leucine-p-nitroanilide (LLNA) were used to determine γ-glutamyl transpeptidase (GGT1) and leucine aminopeptidase (LAP) hydrolytic activity via spectrophotometric monitoring of p-nitroaniline (PNA). EpiKidney tissues were incubated with 2.5 mM of GPNA or 3 mM of LLNA in the presence or absence of 2mM Acivicin for up to 30 min. The reaction was stopped, and hydrolytic activity was determined by absorbance at 405nm.

RNA Extraction and Real-time Quantitative Polymerase Chain Reactions (qPCR): EpiKidney tissues were processed for RNA isolation using a RNAqueous total RNA isolation kit (ThermoFisher). cDNA synthesis was performed on 1 µg of total RNA per sample using a RT^2 First Strand Kit (Qiagen). Individual PrimeTime® qPCR Primers were obtained from IDT (Coralville, Iowa). qPCR was performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad) and quantified using Bio-Rad's CFX Manager Software and SABiosciences' Web-Based PCR Array Data Analysis software.

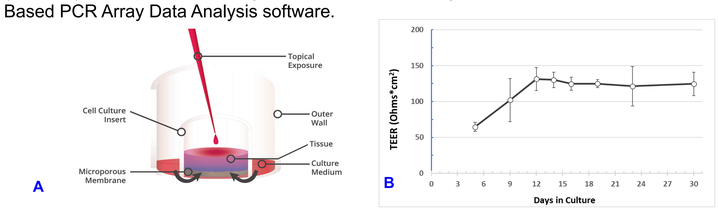

Figure 1: A. Schematic of the EpiKidney tissue model grown in cell culture inserts at air liquid interface (topical tissue surface is exposed to air) allowing for topical or systemic exposure to test materials; **B. Barrier Properties - Transepithelial Electrical Resistance (TEER)** of the EpiKidney tissues at different time points in culture (AVG±SD of 12 lots of tissues).

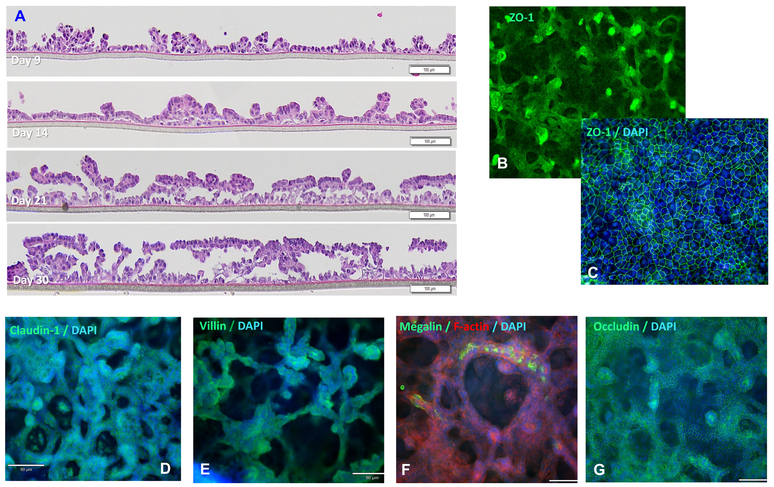

Figure 2: A. H&E-stained histological cross-sections of EpiKidney tissue model at different time in culture; **B-G. Expression of tight junction and brush border proteins** (topical view) by EpiKidney tissues (day 14). Immunohistochemical staining of whole tissues; imaged with ECHO microscope at magnifications: 10X **(B)** and 20X **(C–G)**.

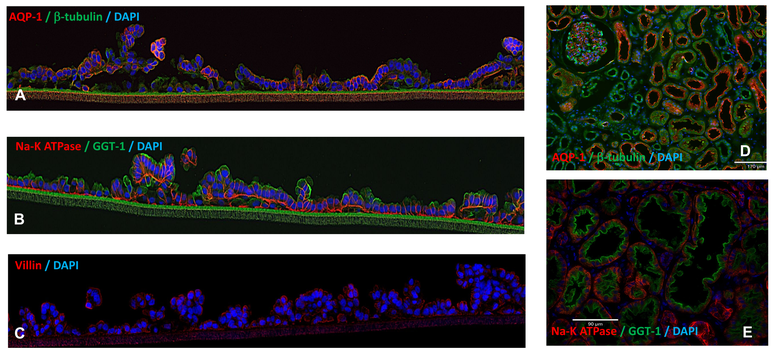

Figure 3: Evaluation of kidney-specific marker expression. Immunohistochemical staining of EpiKidney tissue sections (day 16) (**A-C**) and kidney explant tissue (cortical region) (**D-E**); cross-sections; imaged with ECHO microscope, 10X. Polarized PT epithelium expressing water channel AQP-1, brush border Villin and GGT1 proteins on the apical side and sodium-potassium ATPase pump on the basolateral side.

Gene Name	Protein Name	day 5	day 9	day 12	day 16	day 22	day 30
B2M	B2M						
CLU	CLUSTERIN						
ANPEP	CD13						
ABCC4	MRP4						
SLCO4C1	OATP4C1						
GGT1	GGT1						
SLC22A5	OCTN2						
SLC4A4	HNBC1						
ABCB1	MDR1						
AQP1	AQP1						
SLC22A3	OCT3						
SLC2A9	GLUT9						
SLC22A2	OCT2						
SLC47A1	MATE1						
SLC22A4	OCTN1						
LRP2	MEGALIN						
CYP4A11	CYP450						
SLC5A2	SGLT2						
CUBN	CUBULIN						
ABCC2	MRP2						
ABCG2	BCRP						
SLC34A1	NP2						
SLC22A6	OAT1						
SLC22A7	OAT2						
SLC22A8	OAT3						
SLC5A1	SGLT1						

1.0 < 2^{-dCt} very strong expression
0.1 < 2^{-dCt}< 1.0 strong expression
0.01 < 2^{-dCt}< 0.1 moderate expression
0.001 < 2^{-dCt}< 0.01 low expression
0.0001 < 2^{-dCt}< 0.001 very low expression
not expressed

Figure 4: Evaluation of kidney-specific marker expression. Real-time qPCR analysis (2$^{-\Delta Ct}$) confirmed that EpiKidney tissues express a panel of PTEC-specific markers that are necessary for renal clearance, secretion, and reabsorption. Gene expression was normalized to β2 microglobulin (B2M).

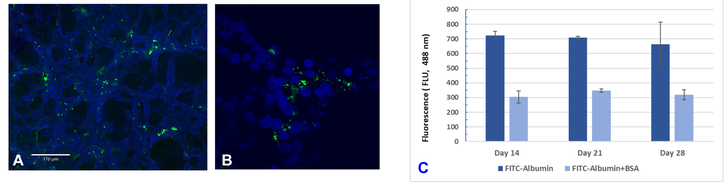

Figure 5: Receptor-mediated FITC-Albumin uptake. A and B. Topical view of the EpiKidney tissues after exposure to FITC-Albumin (2h, topical exposure); imaged with ECHO, 10x **(A)** and confocal microscope, 60x **(B). C.** FITC-albumin absorption was determined at different time in culture. Albumin absorption and the receptor-mediated saturation in the presence of 10 mM of BSA was detected for up to 28 days in culture.

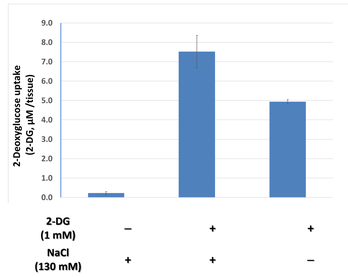

Figure 6: Glucose uptake by EpiKidney tissues. 2-Deoxyglucose (2-DG) uptake was determined on day 19. Data presented as averages of two independent experiments with n=3 tissues per condition. The sodium-glucose symport transport was inhibited in the absence of Na+ ions.

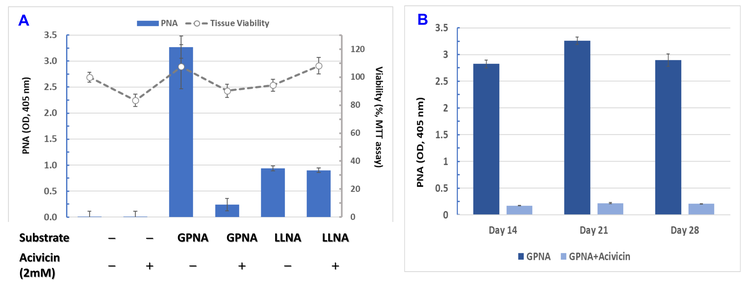

Figure 7: Transpeptidase hydrolytic activity of gamma-glutamyl transpeptidase (GGT-1) and leucine aminopeptidase (LAP) of the EpiKidney tissues was assayed on days 14 to 21. L-γ-Glutamyl-p-nitroanilide (GPNA) and L-leucine-p-nitroanilide (LLNA) were converted to PNA by GGT-1 and LAP enzymes. **A.** GGT-1 activity was inhibited by acivicin, a specific inhibitor of gamma-glutamyl transferase; LAP activity was not inhibited. **B.** GGT-1 activity was present for at least 28 days in culture.

Conclusions

1. EpiKidney tissues:
   - Develop tight tissue barrier (TEER 110.2+/-33.3 Ω·cm^2, **Figure 1B**).
   - Resemble *in vivo* proximal tubular epithelium which is polarized, tubular, and convoluted (**Figures 2-3**)
   - Express tight junction proteins (ZO-1, claudin, occludin) and brush border proteins (villin, megalin, GGT-1) on the apical surface (**Figures 2B-G, 3B**).
   - Express water channel AQP-1 on the apical side and Na-K channel on the basolateral side (**Figures 3A-B**).
2. EpiKidney tissues express PTEC-specific markers that are associated with renal clearance, secretion, and reabsorption (**Figure 4**).
3. EpiKidney tissues performed PT-specific functions:
   - Albumin absorption: receptor-mediated endocytosis was saturable (**Figure 5**).
   - Glucose uptake: inhibition in the absence of sodium ions (**Figure 6**).
   - Transpeptidase hydrolysis by γ-glutamyl transpeptidase (GGT1) and leucine aminopeptidase (LAP): enzyme-specific inhibition (**Figure 7**).
   - ❑ 3D tissues were demonstrated to be functional up to 28 days in culture.
4. EpiKidney model is anticipated to be a valuable tool to improve the predictivity of human responses to pharmacological drug candidates, to study human nephrotoxicity and its mechanisms, and reduce animal usage in pre-clinical drug screening.