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Research Paper

Engineered Kidney Tubules for Modeling Patient-Specific Diseases and Drug Discovery

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ABSTRACT

The lack of engineering systems able to faithfully reproduce complex kidney structures *in vitro* has made it difficult to efficiently model kidney diseases and development. Using polydimethylsiloxane (PDMS) scaffolds and a kidney-derived cell line we developed a system to rapidly engineer custom-made 3D tubules with typical renal epithelial properties. This system was successfully employed to engineer patient-specific tubules, to model polycystic kidney disease (PKD) and test drug efficacy, and to identify a potential new pharmacological treatment. By optimizing our system we constructed functional ureteric bud (UB)-like tubules from human induced pluripotent stem cells (iPSCs), and identified a combination of growth factors that induces budding morphogenesis like embryonic kidneys do. Finally, we applied this assay to investigate budding defects in UB-like tubules derived from a patient with a *PAX2* mutation.
Our system enables the modeling of human kidney disease and development, drug testing and discovery, and lays the groundwork for engineering anatomically correct kidney tissues *in vitro* and developing personalized medicine applications.

1. Introduction

The ability to engineer key functional units of living kidneys *in vitro* faithfully and reproducibly could revolutionize experimental nephrology and pharmacology [1, 2]. Engineered tissues could be used to investigate the basic processes underlying kidney development, function and disease, for drug discovery and toxicology studies, and potentially even as therapeutic replacements for diseased organs. To this end, a plethora of 3D culture systems have been developed over the last 50 years, using embryonic kidney cells [3–5], tissue fragments [6, 7], and primary cells or immortalized cell lines [8–13], as well as whole proximal tubules [14], and these are now considered classical tools for studying kidney development and pathophysiology. However, despite producing epithelial structures that are somewhat similar to their *in vivo* counterparts, most methods still have significant limitations. Because they rely exclusively on cell-driven self-assembly and are not controlled spatiotemporally, the resulting structures are heterogeneous in size, shape and composition. Other drawbacks include the need for extensive cell culturing, ranging from several days to weeks [9, 11] and the presence of other cell types [9], tissues [7, 12] or conditioned media [11], all of which negatively affect the reproducibility, cost-effectiveness, and overall applicability of these methods. Finally, these methods have not been tested using human cells, which is essential for studying human kidney organogenesis and pathophysiology.

Recently, the generation of kidney organoids, starting with human induced pluripotent stem cells (iPSCs), has created significant opportunities for disease modeling and toxicology studies in human tissue [15–19]. However, the usefulness of organoids generated through self-organization processes is still limited by major technical problems. One is that engineering methods cannot accurately replicate organogenesis *in vitro*, due to the kidney's intricate morphology and the multiple interactions between different cell lineages -of renal and extra-renal origin- during kidney development. In fact, several anatomical deficiencies are present in self-organizing tissues, such as the complete lack of the ureteric epithelium or random formation of ureteric buds (UB), nephron-nephron connections and branching nephrons [16, 17, 19]. Although important steps toward generating more realistic kidney

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organoids have recently been made by making branch-competent UB [20, 21], tissues that can be applied in biomedical research have not been generated yet. For the moment, apart from toxicity tests, there is no engineering technology that can be used to model human kidney disease quantitatively and test therapeutic drug efficacy. One possible approach to bridge this methodological gap would be to directly engineer kidney-specific units with predefined shapes and cell types that could be used for biomedical screening or combined with organoid technologies to generate more realistic mini-organs.

Engineering systems with these abilities would be particularly useful for personalized drug testing and studying human diseases that affect developmental processes, which cannot currently be studied because of the scarcity of suitable human samples.

To meet these methodological needs we developed a practical 3D engineering system that enables the generation of custom-made kidney tubules, which can actually be used for disease modeling, drug testing, and studying human kidney development. First, using 3D printing technology and PDMS prototyping, we fabricated single, branched and fractal-like moulds, into which we seeded MDCK cells to grow into tubules, with structural and functional features typical of renal epithelium. By inducing cyst formation in engineered tubules pharmacologically, we modeled polycystic kidney disease (PKD) and tested different drugs' effects on cyst regression, demonstrating that 2-deoxy-D-glucose (2DG) and berberine -a compound never tested in PKD models before- displayed a robust effect on cyst regression and restored tubular lumen and epithelial organization. To confirm that our setting can be used for pre-clinical testing in human disease, we engineered tubules using epithelial cells that were derived from an autosomal dominant polycystic kidney disease (ADPKD) patient and demonstrated that 2DG and berberine had a strong anticystogenic effect in patient-specific polycystic tubules.

By further optimizing our original protocol, we generated functional human UB-like tubules using different iPSC lines that could grow *in vitro* and undergo budding morphogenesis when co-cultured with embryonic kidneys. Finally, we identified a combination of growth factors that induces human tubules to bud and ramify, and applied this assay to study developmental defects in tubules derived from a patient carrying a heterozygous *PAX2* mutation.

This engineering system is a robust, quick and efficient tool for generating custom-made, complex functional tubules, modeling PKD, and discovering new drugs, and is also useful for studying human kidney development and individual patient's genetic defects. It also lays a solid methodological groundwork for engineering anatomically correct human kidney tissues or organoids *in vitro*.

2. Materials and Methods

2.1. PDMS Scaffold Fabrication

Polydimethylsiloxane (PDMS) scaffolds (Sylgard 184 Silicone elastomer kit, Dow Corning, Midland, MI) were fabricated using a 3-step prototyping approach: design, 3D printing and replica molding. First, scaffolds were designed by using Computer Aided Design (CAD) software (Autodesk 123D® Design ©2014 Autodesk Inc.) with different geometries: (i) three scaffolds with width and depth of 1 mm: a linear one with a straight cavity, a bifurcated one with terminal 80° branching and an asymmetrical one with two 30° lateral branches extending from the central trunk; (ii) two more complex ramified and tree-like scaffolds, the latter containing a fractal-like pattern, both 0.7 mm in depth, and ranging in width from 0.7 to 0.5 mm, respectively; (iii) a multichannel scaffold containing 11 linear microchannels that were 0.4 mm in depth × 0.4 mm in width × 9 mm in length, within a miniaturized culture chamber (1 mm in depth).

3D printing technology (Professional Pico Plus39 stereolithographic 3D printer, Asiga, CA, USA) was applied to build plastic masters (Asiga PlasWhite photopolymer resin). The printer uses an upside-down system with nominal XY pixel resolution down to 39 μm and servo resolution of 250 nm. The masters were printed at 0.5 s of exposure time, with a printing speed of 0.33 cm/h and 10 μm vertical step size. After printing, masters were cleaned by rinsing with isopropyl alcohol for 5 min, washed in distilled water, dried and exposed to UV light (365 nm) (Bio-Link-BLX-365 nm, 80 W, Vilber Lourmat) for 40 min to complete curing. To facilitate the de-molding of the polymeric replicas, masters were functionalized with an oxygen plasma treatment (Tucano plasma reactor Gambetti Kenologia) and with the deposition, from vapor phase, of an anti-sticking layer of Trichloro (1H,1H,2H,2H-perfluorooctyl) silane (FOTS, 448931-10G, Sigma-Aldrich, Saint Louis, Missouri, USA). Subsequent REplica Molding (REM) steps provide several polymeric replicas starting from a single master as previously described [22]. The PDMS base and curing agent (10:1 w/w) mixture (PDMS prepolymer) was poured onto the 3D-printed masters. PDMS was then degassed using a vacuum desiccator for 20 min, and cured in an oven at 60 °C for 2 h. Next, the cured PDMS was peeled off from the master resulting in a freestanding perforated PDMS stencil (with the pattern of the master). To control the thickness of the bottom layer of the scaffold, the PDMS stencil was placed on a 20 μm-thick PDMS prepolymer layer spin-coated on a glass cover slip, and then bonded together by thermal curing. The PDMS scaffold was obtained by detaching the assembled PDMS from the glass slide. This procedure results in scaffolds with intruded patterns, open at the top, in the form of linear, ramified, and fractal-like channels with rectangular cross-sectional geometry. Before use, PDMS scaffolds are placed in a Petri dish, with the open side facing up. To take advantage of the optical properties of PDMS for microscopy [23] and enable imaging of the forming tubules during culture inside the PDMS scaffold, the thickness of the bottom layer was maintained around 20 μm. Scaffold hydrophobicity was preserved to avoid cellular attachment [24, 25] and facilitate tissue recovery from scaffolds. Scaffolds were re-used multiple times after standard sterilization.

2.2. Cell Line and Human Primary Cell Culture Conditions

Madin-Darby Canine Kidney (MDCK) type II cell line (Cat#00062107, RRID:CVCL_0424, European Collection of Cell Cultures, Salisbury, UK) was maintained in Minimum Essential Medium Eagle (MEM) (Cat#M5650, Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS) (Cat#10270, Invitrogen Corporation, Carlsbad, CA, USA), 1% L-glutamine (Cat#25030024, Invitrogen), 1% penicillin and streptomycin (Pen-Strep) (Cat#15140122, Invitrogen), and cultured in standard conditions (37 °C, 5% CO_2, 20% O_2). When 80–90% confluent, MDCK were harvested using 0.25% (w/v) Trypsin–0.53 mM EDTA (25,200,056; Invitrogen) for 2 min at 37 °C and seeded at a dilution of 1:8.

Primary single cyst-derived huADPKD cells isolated from an individual donor patient were purchased from Discovery BioMed (Discovery BioMed Inc., Birmingham, AL, USA) and cultured following the provider's recommendations. Briefly, huADKD cells were grown on permeable, clear polyester filter supports (Cat#3450, Corning) in DBM RenalCyte Specialty Medium (Discovery BioMed Inc.). When 90% confluent, huADPKD cells were harvested using 0.05% (1×) Trypsin-EDTA (Cat#15400054, Invitrogen) for 2 min at 37 °C and seeded at a dilution of 1:6. Culture medium was changed every 3 days. The huADPKD cells were used for no >6 passages.

Human iPSC lines used in our experiments were derived from healthy donor somatic cells by different technologies and characterized as previously described [26, 27]. Specifically, iPSC clone IV (RRID:CVCL_IT61) was obtained from neonatal fibroblasts using STEMCCA lentivirus [27] and iPSC#16 cells were derived from peripheral blood mononuclear cells (PBMCs) through non-integrative Sendai virus [26]. Cells were maintained in mTeSR1 (Cat#05850, StemCell Technologies, Vancouver, Canada) enriched with mTeSR1 5× Supplement (StemCell Technologies) and cultured in standard conditions (37 °C, 5% CO_2, 20% O_2) on Matrigel hESC-qualified Matrix (Cat#354277, Corning, NY, USA)-coated

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