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Heparin-based hydrogels induce human renal tubulogenesis *in vitro*

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ABSTRACT

Dialysis or kidney transplantation is the only therapeutic option for end stage renal disease. Accordingly, there is a large unmet clinical need for new causative therapeutic treatments. Obtaining robust models that mimic the complex nature of the human kidney is a critical step in the development of new therapeutic strategies. Here we establish a synthetic *in vitro* human renal tubulogenesis model based on a tunable glycosaminoglycan-hydrogel platform. In this system, renal tubulogenesis can be modulated by the adjustment of hydrogel mechanics and degradability, growth factor signaling, and the presence of insoluble adhesion cues, potentially providing new insights for regenerative therapy. Different hydrogel properties were systematically investigated for their ability to regulate renal tubulogenesis. Hydrogels based on heparin and matrix metalloproteinase cleavable peptide linker units were found to induce the morphogenesis of single human proximal tubule epithelial cells into physiologically sized tubule structures. The generated tubules display polarization markers, extracellular matrix components, and organic anion transport functions of the *in vivo* renal proximal tubule and respond to nephrotoxins comparable to the human clinical response. The established hydrogel-based human renal tubulogenesis model is thus considered highly valuable for renal regenerative medicine and personalized nephrotoxicity studies.

Statement of Significance

The only cure for end stage kidney disease is kidney transplantation. Hence, there is a huge need for reliable human kidney models to study renal regeneration and establish alternative treatments. Here we show the development and application of an *in vitro* human renal tubulogenesis model using heparin-based hydrogels. To the best of our knowledge, this is the first system where human renal tubulogenesis can be monitored from single cells to physiologically sized tubule structures in a tunable hydrogel system. To validate the efficacy of our model as a drug toxicity platform, a chemotherapy drug was incubated with the model, resulting in a drug response similar to human clinical pathology. The established model could have wide applications in the field of nephrotoxicity and renal regenerative medicine and offer a reliable alternative to animal models.

1. Introduction

More than 20 million people in the United States alone are estimated to suffer from some degree of chronic kidney disease (CKD) [1]. CKD is a progressive loss of renal function that can lead to end stage renal disease (ESRD), a condition where the kidneys retain less than 10–15 percent of their normal function. The only current treatment for ESRD is dialysis or kidney transplantation, both of which have immense limitations. Therefore, there is a critical need for new therapeutic treatments such as regenerative therapies. Despite ongoing research into the regenerative capabilities of the kidney, much is still unknown about regenerating the nephrons, the functional units of the kidney [2–4]. Specifically, highest interest is focused on the renewal of proximal tubules because they are the region most targeted by renal pathologies and drug-induced injury. However, an *in vitro* renal tubulogenesis model to recapitulate the complex physiology of the human kidney in an adaptable system conducive for regenerative therapy testing, has yet to be established. The ideal assay would mimic the human 3D structure and function of proximal tubules in a well-defined, tunable, robust

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matrix that can be easily analyzed in high throughput screenings for regenerative medicine, developmental, or toxicity applications.

Recent progress in the generation of kidney organoids developed from human stem cells can be considered highly promising for creating more realistic human *in vitro* models [5–12]. Organoids utilizing induced pluripotent stem cells have great potential in personalized medicine, but are limited due to the retention of epigenetic characteristics of their somatic tissue of origin [13]. This feature could affect their differentiation ability and account for the lower differentiation efficiency of induced pluripotent stem cells to form tubular organoids compared to human embryonic stem cells [7]. Alternatively, embryonic stem cells have high differentiation purity, but they cannot be used in personalized medicine and ethical aspects restrict their application. In addition, pluripotent stem cells, in general, carry the risk of teratoma formations *in vivo* and can lead to a distribution of non-renal cells *in vitro* [7,14,15]. For these reasons, the application of primary renal tubule cells is currently considered the most promising option for nephrotoxicity assays, as well as for the exploration of new therapeutic strategies for kidney disease.

Previously described 3D renal tubulogenesis models use reconstituted collagen I, Matrigel™, or a combination of these materials [16–27]. Interestingly, Rosines et al. found that when aggregates of mouse inner medullary collecting duct cells (IMCD) were cultured in Matrigel, they formed a tubule that resembled the ureteric bud of the developing kidney, suggesting that 3D matrices are necessary to induce renal tubulogenesis *in vitro* [28]. Furthermore, using immortalized human renal cortical epithelial cells, DesRochers et al. created a 3D tubule model in Matrigel/collagen I and systematically compared nephrotoxicity outcomes in 3D versus 2D cell culture [29,30]. They reported better overall function of cells in 3D and found that the 3D model was more sensitive to nephrotoxins, supporting the belief that 3D models are a better representation of the *in vivo* environment. While these matrices support the growth of renal cells, the formed tubular structures do not accurately portray the size or structure of the human proximal tubule. In addition, batch-to-batch variations of the matrix materials cause difficulties in handling and reproducibility of the cultures, making them suboptimal for high throughput screening. Furthermore, Matrigel is derived from animal sarcoma cells and is, therefore, unsuitable for human clinical applications [31]. Moreover, these animal-derived hydrogels lack tunability in mechanical and biofunctional properties, which is required for testing the impact of exogenous signals on renal tubulogenesis for exploring therapeutic treatments.

Synthetic matrices could circumvent these problems by offering controlled, tunable material characteristics that can be customized to stimulate renal tubulogenesis. Astashkina et al. successfully used a synthetic matrix made of hyaluronic acid and poly(ethylene glycol) diacrylate (PEGDA) to embed mouse tubules in a 3D model for nephrotoxicity [32,33]. This model showed the efficacy of 3D models for high throughput nephrotoxicity assays. However, the method is not clinically relevant for humans and does not allow the monitoring of tubulogenesis from single cells to tubule structures.

The aim of our study was to develop a tunable 3D hydrogel model where renal tubulogenesis and nephrotoxicity can be reliably studied in a well-defined system. We examined a range of hydrogels based on heparin, a highly sulfated glycosaminoglycan, and four-armed poly(ethylene glycol) (starPEG), with varying biophysical and biomolecular properties (Fig. 1). Heparin was used as a building block of the polymer networks because of its ability to bind and stabilize a variety of growth factors and proteins [34,35]. Matrix metalloproteinase (MMP) cleavable peptide sequences were also incorporated as linker units to allow for cell-driven remodeling of the matrices [36]. As comprehensively investigated before in theoretical and experimental studies, the physical and biomolecular properties of these hydrogels can be independently tuned by simply changing the ratio of the components, providing a versatile toolbox to study renal tubulogenesis [37,38]. Among the tested hydrogel variants, a soft matrix consisting of heparin crosslinked to starPEG with a MMP cleavable sequence, led to tubular structures that display the morphology and function of the human *in vivo* renal proximal tubule. This method was also successfully translated to the culture of primary human proximal tubule epithelial cells, creating valuable options for patient-specific renal toxicity testing. Taken together, the established hydrogel-based *in vitro* human tubulogenesis model has broad applicability in the field of drug toxicity, disease modeling, and regenerative medicine.

2. Materials and methods

2.1. Cell culture

HK-2 (human kidney-2) cells were obtained from American Type Culture Collection (ATCC, #CRL-2190) where they were derived from normal adult kidney and immortalized by transduction with human papilloma virus 16 (HPV-16) E6/E7 genes. HK-2 cells were cultured in DMEM/F-12 media (Gibco) supplemented with 10% fetal bovine serum (FBS, Biochrom) and 1% penicillin/streptomycin (P/S) solution. Conditionally immortalized proximal tubule epithelial cells (ciPTEC) were generously donated by Prof. Roos Masereeuw (Utrecht University, Utrecht, The Netherlands) and Dr. Martijn Wilmer (Radboud university medical center, Nijmegen, The Netherlands). CiPTEC were isolated from urinary sediment of a healthy donor and immortalized with SV40T and hTERT, as previously described [39]. CiPTEC were grown for proliferation at 33°C in DMEM-Ham's F-12 media(Gibco) phenol red free, supplemented with 5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), 5 ng/ml selenium (Sigma), 36 ng/ml hydrocortisone (Sigma), 10 ng/ml EGF (Sigma), 40 pg/ml tri-iodothyronine (Sigma), and 10% FBS. For maturation, ciPTEC were transferred to 37°C. Primary human renal proximal tubule epithelial cells were purchased from ATCC (#PCS-400-010). Primary proximal tubule cells were isolated from a normal human kidney through tissue dissection and differential trypsinization and cultured in the same media as ciPTEC, with the exception of 0.5% FBS instead of 10%. Primary proximal tubule cells were used for experiments at passages 2–4. Culture medium was changed every 2–3 days for all cell experiments.

2.2. Three-dimensional hydrogel culture

Hydrogels were fabricated as previously described [37]. All hydrogel material was dissolved in phosphate buffered saline (PBS) with 1% P/S. In brief, heparin-maleimide was dissolved in ¼ of the total gel volume. Proximal tubule cells were then added to the heparin solution in ¼ of the total gel volume to make 2×10^6 cells/ml (50,000 cells/gel). StarPEG or starPEG-MMP was dissolved in ½ of the total gel volume. The cell/heparin solution was then mixed with the starPEG solution to form a 25 μl hydrogel. For all starPEG-based hydrogels, a 25 μl gel was cast onto a microscope slide coated with Sigmacote® (Sigma) for 20 min. After polymerization, the gels were removed from the slide and placed in a 24 well plate with warm media where they were free floating throughout the culture.

Degradable starPEG-MMP-starPEG gels were formed in a similar way by dissolving starPEG-MMP in ¼ of the total gel volume and then mixing it with the cells. Maleimide terminated starPEG (starPEG-mal) was dissolved in ½ of the total gel volume and then