A starPEG-heparin hydrogel model of renal tubulogenesis

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Abstract

Currently, the only treatment for end stage renal disease is dialysis or kidney transplantation. These methods contain obvious limitations such as the palliative nature of dialysis treatment and the lack of available organs for transplantation. As a result, there is a dire unmet need for alternative options. Regenerative therapies that focus on stimulating the regrowth of injured tissue can be a promising alternative. A critical step in the development of such therapeutic remedies is obtaining robust models that mimic the complex nature of the human kidney. The proximal tubules are a particular region of interest due to their important role in reabsorption and secretion of the glomerular filtrate and the blood, making them particularly susceptible to nephrotoxicity and renal pathologies. For this reason, the goal of this thesis was to engineer a 3D model of human proximal tubulogenesis that would allow for both developmental and regenerative studies. The ideal assay would mimic the human 3D structure and function of proximal tubules in a tunable, robust matrix that can be easily analyzed in throughput screenings for regenerative medicine and toxicity applications.

In this thesis, we show the development, characterization, and application of an in vitro human renal tubulogenesis model using a modular and tunable biohybrid starPEG-heparin hydrogel platform. A range of hydrogel mechanics and compositions were systematically tested to determine the optimal conditions for renal tubulogenesis. The results revealed that only soft hydrogels based on heparin and matrix metalloproteinase (MMP) enzymatically cleavable crosslinkers led to the generation of polarized tubule structures. The generated tubules display polarization markers, extracellular matrix components, and organic anion transport functions which mimic the human renal proximal tubule. To the best of our knowledge, this is the first system where human renal tubulogenesis can be monitored ex vivo from single cells to physiologically sized tubule structures in a 3D tunable matrix. Moreover, it was found that heparin played a role in the polarization of proximal tubule cells in the hydrogel culture.

The established starPEG-MMP-heparin based hydrogel model was then tested for its application as a renal tubulogenesis model by the addition of pro and anti-tubulogenic factors. It was found that the addition of growth factors and MMP inhibitors could promote and inhibit tubulogenesis, respectively. This model can be used to modulate tubulogenesis by adjusting the mechanical
properties of the hydrogel, growth factor signaling, and the presence of insoluble cues (such as adhesion peptides), potentially providing new insights for regenerative therapy.

To examine if the established hydrogel-based renal tubulogenesis model could be applied as a drug toxicity platform, the nephrotoxic, chemotherapeutic drug, cisplatin was incubated with the renal tubule model. The tubular structures showed a dose-dependent drug response resembling the human clinical renal pathology. The injured tubular structures also expressed the early in vivo proximal tubule injury biomarker, kidney injury molecule-1 (KIM-1).

In conclusion, a hydrogel-based renal tubulogenesis model was successfully developed, characterized, and applied as a nephrotoxicity assay. Our findings suggest that the established hydrogel-based model can additionally be used for personalized medicine, where a patient’s predisposition to drug-induced renal injury or specific renal regenerative medicine treatments could be examined. This platform provides a novel approach to study human nephrotoxicity and renal regenerative medicine ex vivo, limiting the need for animal models, and potentially paving the way for more reliable preclinical trials.
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Chapter 1

Introduction

1.1 Motivation

It is estimated that one in ten American adults currently suffer from some degree of chronic kidney disease (CKD)\(^1\). CKD is a progressive loss of kidney function that in early phases can often go unnoticed. Without treatment, CKD can progress to end stage renal disease, a condition where the kidneys retain less that 10-15 percent of their normal function. The only treatment options for ESRD are dialysis, a palliative treatment, or kidney transplantation.

More people are waiting for kidney transplants than any other organ. In the United States alone, more than 99,000 people are currently on the waiting list for a kidney transplant\(^2\). Only a small amount, less than 1/3, will receive a kidney. In addition to recovering from the invasive surgery, they will also have the burden of taking immunosuppressant medication for the rest of their lives to decrease the chance of kidney rejection. This medication will make them more susceptible to infections, high blood pressure, weight gain, and can even increase the risk of some types of cancer\(^3\). Thus, the current treatment options for ESRD are immensely unsatisfactory.

Regenerative medicine is a promising alternative to the current therapeutic treatment options of CKD and ESRD. Regenerative medicine therapies use the body’s own mechanisms to recreate the injured sites\(^4\). In this way, tissues and organs can be rebuilt using normal physiological processes and potentially provide a cure, rather than a palliative treatment. Despite ongoing research into the regenerative capabilities of the kidney, much is still unknown about regenerating the nephrons, the functional units of the kidney\(^5\)–\(^7\). The proximal tubules are the region most targeted by renal pathologies and drug-induced injury and consequently much interest is focused on the renewal of these structures. In vivo studies of the proximal tubule are limited due to the complex anatomy of the kidney, which makes visualization of specific functions complicated.

To establish novel regenerative renal therapies, a reliable model of the human kidney or human proximal tubule is needed. To date, an in vitro renal tubule model that fully recapitulates the complex
3D physiology of the kidney has not yet been created. As a result, animal models and 2D cell culture models are often used in preclinical trials. However, results from animal models contain interspecies differences. For example, the kidneys are a major target of drug-induced toxicity and a limited number of animal experiments accurately predict the human response to drugs. As a consequence, adverse reactions are often not found until human clinical trials. Alternatively, cells grown on conventional 2D tissue culture substrates lack the 3D cell-cell and cell-matrix interactions that are crucial in maintaining cell phenotype and function, and therefore, are not accurate representations. For these reasons, 3D models have recently been gaining interest as promising approaches to generate more realistic renal models.

1.1 Objectives

The ideal renal model should recapitulate the human 3D structure and function of the kidney, especially the proximal tubules which play a crucial role in maintaining homeostasis and toxin excretion. The model should also be robust and easily analyzed so that it can be used in throughput screenings for applications in regenerative medicine and toxicity. The aim of this study was to establish a 3D renal proximal tubulogenesis model using starPEG-heparin hydrogels.

A renal tubule model should replicate in vivo renal tubulogenesis so that this process can be studied for renal regenerative medicine. Thus, the matrix should be tunable, allowing for the adjustment of mechanical and biomolecular factors to dissect their effects on tubulogenesis. Another important application for a renal tubulogenesis model is as a nephrotoxicity assay because the proximal tubule is a major target of drug toxicity. The generated tubules could be incubated with potential drug candidates to assess their toxicity to the kidney. Additionally, the model could be used as a renal disease model or as a source of cells for therapy.

To accomplish the goal of creating an in vitro 3D renal tubulogenesis model, the project was broken down into six main objectives:

1.) Evaluate the conditions to induce tubule formation in biohybrid starPEG-heparin hydrogels.

2.) Characterize the renal structures produced in different hydrogel variants to determine the condition that best replicates the physiological human tubule.
3.) Examine the impact of soluble cues on the renal tubulogenesis model.

4.) Evaluate functionality of the tubulogenesis model by examining the function of organic transporters, which are important for the removal of potentially toxic drugs, metabolic wastes, and drug metabolites.

5.) Investigate the application of the model for \textit{ex vivo} renal tubulogenesis using primary human proximal tubule epithelial cells.

6.) Probe the potential of the model as a human nephrotoxicity assay by analyzing the response to the nephrotoxin, cisplatin.
Chapter 2

Fundamentals

This chapter will provide the framework of information needed to understand the scope of this thesis. First, the kidney physiology is briefly introduced. Then the anatomy of the nephrons, the functional units of the kidney, is presented. Next, the function and structure of the proximal tubule, an invaluable part of the nephron, are discussed, followed by what is currently known about renal tubulogenesis. The next section describes the kidneys susceptibility to drug toxicity. Finally, the advantages and disadvantages of the current state of in vitro biomaterial-based renal models are discussed and starPEG-heparin hydrogels are proposed as a promising alternative.

2.1 Kidney physiology

The kidney is a complex organ that consists of more than 20 different specialized cell types and plays a critical role in maintaining body fluid homeostasis. The kidney receives about 25% of cardiac output, roughly 180 liters of blood daily which it filters for toxins, metabolic wastes, and ions. Blood enters the kidney through the renal artery, where it is heavily filtered and then removed through the renal vein. The kidney also regulates the body’s fluid levels of electrolytes, and produces hormones to maintain blood pressure and healthy bones\(^1\). Specifically, the kidney yields the hormones renin, erythropoietin, vitamin D, and prostaglandins\(^2\).

The complete renal system consists of two kidneys located on each side of the spine, beside the posterior wall of the abdomen. Each kidney is enclosed by a fibrous capsule made of a dense network of primarily collagen and elastin fibers; this serves as protection to the kidney\(^3\). Under the kidney capsule, the renal parenchyma can be broken down laterally into conical lobes termed renal pyramids that consist of an outer region called the cortex, an outer and inner medulla, and the papilla. The papilla is the location where urine drains into the minor and major calyces and eventually to the ureter, where it is then secreted into the bladder\(^4\).

During development, the human kidney is formed from the intermediate mesoderm (IM), located between the paraxial mesoderm and the lateral plate mesoderm in the developing embryo. From this
tissue, the ureteric bud (UB) and metanephric mesenchyme (MM) are formed. A complex interplay between these two structures induces UB to undergo branching morphogenesis and the MM to undergo mesenchymal-to-epithelial transition, which produces a polarized epithelial structure. This polarized epithelial structure is termed the renal vesicle and it develops into a comma-shape, and then a S-shaped body with cells that eventually differentiate into the specialized sections of the nephron\textsuperscript{15}.

The following chapter gives an overview of the nephron and its importance in the kidney, with an emphasis on the proximal tubule and the previous models to recreate these structures \textit{in vitro}.

### 2.1.1 Nephron anatomy

The average adult human kidney contains around 1 million nephrons, which are responsible for the vital functions of the kidney. In humans, nephrogenesis is fully completed intrauterine, meaning that new nephrons do not form during an individual’s life\textsuperscript{16}. The nephron consists of two parts, the renal corpuscle and the renal tubule. Nephrons are formed from the renal vesicle that develops into an S-shaped body. The lower (proximal) limb of the S-shaped body develops into the renal corpuscle and the distal end forms the renal tubules\textsuperscript{17} (Fig. 2.1.1).

The renal corpuscle contains the glomerulus, Bowman’s space, and Bowman’s capsule and is formed when the initial end of the nephron encapsulates a mass of capillaries, known as the glomerulus. The glomerulus is essentially a tuft of capillaries that protrudes into the Bowman’s space (urinary space) and is surrounded by the Bowman’s capsule. The renal corpuscle contains three different types of cells: mesangial cells, endothelial cells, and the specialized epithelial cells, podocytes. The specialized capillaries are held together by the mesangial cells and this section is covered by the glomerular basement membrane (GBM) followed by a layer of podocytes on the outer surface\textsuperscript{18}. The glomerulus filters the blood brought in by the capillaries for macromolecular molecules, allowing only molecules with a molecular weight less than 70 kDa to pass through the filter\textsuperscript{19}. The charge, shape, and flexibility of the molecule also influence its ability to be cleared from the glomerulus. The resulting filtrate is then collected in the Bowman’s space, which opens into the tubular lumen, and later into the collecting ducts (Fig. 2.1.1.). Non-filtered blood components such as blood cells will leave the glomerulus through the efferent arteriole.
Figure 2.1. **Kidney and nephron anatomy.** Approximately 1 million nephrons construct the average human kidney. The nephron can be divided into the following sections: glomerulus, proximal tubule, loop of Henle, distal tubule, and the collecting duct. The proximal tubules play an important part in reabsorption and secretion of important factors in the blood and filtrate and will be the focus of the thesis. Adapted from Sanchez-Romero et al. Copyright permission granted from Elsevier Publishing Group.

At the urinary pole, the Bowman’s capsule turns into the epithelium of the tubule region of the nephron. The tubule is characterized by different segments pertaining to their specialized secretion and absorption functions (Fig. 2.1.2). The first section is the proximal tubule which has a simple cuboidal epithelium and is responsible for reabsorbing the majority of the filtrate (Fig. 2.1.2). For this reason, the proximal tubule contains a dense brush border made up of microvilli to enlarge the absorption area. The majority of the proximal tubule resides in the renal cortex of the kidney. The proximal tubule then lowers into the renal medulla where the cells obtain a flatter morphology with less microvilli, and become the Loop of Henle.

The Loop of Henle can be divided into three parts, the descending thin limb, the ascending thin limb, and the thick ascending limb (Fig. 2.1.2). The thin limbs are composed of simple squamous epithelia that are 15-30 μm in diameter and have a cell size of 1-5 μm and the thick ascending limb consists of cuboidal epithelia. The different segments have distinct functions based on the overall...
role of the loop to reabsorb salt (NaCl) and water. The descending limb is freely permeable to water, thereby being the location of water reabsorption, whereas the ascending limbs are less permeable to water and the site of reabsorption of sodium chloride (NaCl)\textsuperscript{24} (Fig. 2.1.2). The thick ascending loop returns to the renal cortex and transforms into the distal convoluted tubule which regulates sodium, potassium, and divalent cation homeostasis\textsuperscript{25} (Fig. 2.1.2). The distal tubules from several nephrons converge onto a single collecting duct and the accumulation of collecting ducts form the renal papilla. From the collecting duct, the filtrate travels into the ureter and then to the bladder where it is later excreted from the body as urine.

Figure 2.1.2. Summary of the functions of each region of the nephron. The glomerular filtrate travels through the nephron and is eventually excreted from the body as urine. Adapted from DesRochers et al.\textsuperscript{26}

2.1.2 Proximal tubule ultrastructure and function

Proximal tubules play a crucial role in the function of the nephron. They are responsible for reabsorbing approximately 65% of the filtrate including amino acids, low molecular weight proteins, salt, potassium, and water\textsuperscript{27}. They also regulate the acid-base balance in the body by reabsorbing 80% of the filtered bicarbonates and reabsorb glucose to maintain glucose metabolism\textsuperscript{27,28}. The differences between mammalian proximal tubule cells among species is mostly based on size and amount of cell
organelles\textsuperscript{20}. As all of the experiments in this thesis pertain to human cells, the following section will focus on the human proximal tubule.

The human proximal tubule is about 10-14 mm long and 30-60 μm wide and can be divided into different segments, the convoluted tubule (called S1 and S2) and the straight tubule (referred to as the S3 segment)\textsuperscript{23,30}. Each segment has a distinct morphology to fulfill their physiological roles. Proximal tubules consist of cuboidal epithelial cells that have an apical basal polarity\textsuperscript{21}. The apical side of the cells faces the lumen of the tubule and contains a dense border of microvilli termed the brush border (Fig. 2.1.3). The brush border creates a larger surface area to allow for more cell adsorption, a key role of the proximal tubules.

Each proximal tubule cell has a single primary cilium on the apical surface that senses changes in luminal flow rate and circumferential stretch\textsuperscript{21,31}. Inside the cell, the Golgi apparatus is located near the apical membrane and the round nuclei are usually situated near the cell base or centrally\textsuperscript{23,30} (Fig. 2.1.3). The basal membrane is partitioned into interdigitating basolateral folds which enclose mitochondria (Fig. 2.1.3A). The cells are attached to neighboring cells through junctional complexes near the apical surface, specifically the tight junction (zonula occludens, ZO-1) and the intermediate junction (zonula adherens) (Fig. 2.1.3A). Tight junctions are important for preserving the barrier between the lumen of the tubule and the interstitium. However, some disruptions in tight junctions allow certain molecules to be reabsorbed through a paracellular path\textsuperscript{30}.

The S1 segment of the proximal tubule is the region directly in contact with the renal corpuscle. Proximal tubule cells in this region have a greater density of microvilli, deeper basolateral membrane infoldings, and a higher amount of mitochondria to provide energy for solute transport. The S1 segment also shows extensive endocytosis near the apical membrane consisting of endosomes, vesicles, and invaginations (Fig. 2.1.3). In addition, many lysosomes are available to process and degrade the incoming material. The later segments (S2 and S3) have microvilli that are shorter and sparser (e.g. less surface area) and less mitochondria, indicating that less reabsorption occurs in these segments\textsuperscript{30}. The later segments also have tighter junctional complexes, making them less permeable to solutes and fluid\textsuperscript{32}. 
Figure 2.1.3. Morphology of the proximal tubule epithelial cell. A) Schematic of a cuboidal proximal tubule cell. Adapted from Krstic\textsuperscript{23}. Copyright permission granted by Springer Publishing Group. B) TEM micrograph of a human proximal tubule cell. Adapted from Møller et al.\textsuperscript{33}. Mv presents microvilli which make up the brush border (BB) of the cell. L represents lysosomes, CV and V are condensing vesicles, G is the Golgi apparatus, M are the mitochondria, N is the nucleus, BL is the basal lamina with BLa being the basal labyrinth that is formed from the basal invaginations (Inv). S are saccules, P are cell processes, J are the junctional complexes that connect neighboring cells, and Pr are sheet-like processes that interlock with processes from neighboring cells. C refers to a peritubular capillary (C).

The base of the proximal tubule cells is attached to the basement lamina which is a thin sheet of extracellular matrix that coats the outer surface of the proximal tubule and part of the tubular basement membrane. The main components of the renal proximal tubule basement membrane are laminin-1, laminin-10, collagen IV, entactin/nidogen, perlecan, and bamacan. The composition of the basement membrane is important for the function of the proximal tubule since they are continuously absorbing and secreting molecules from the surrounding peritubular capillaries.

The proximal tubule is the first region of the tubule that the glomerular filtrate enters after ultrafiltration by the glomerulus. Therefore, it is the location where most of the reabsorption of the glomerular filtrate takes place. As the glomerulus filters high molecular weight proteins, the proximal tubule filters the remaining proteins and molecules out of the filtrate through endocytosis, namely, peptide hormones and albumin are reabsorbed. They also reabsorb amino acids, low molecular weight proteins, salt, potassium, and water. The majority of solute and water reabsorption in the proximal tubule takes place in an isotonic manner causing minimal changes in luminal osmolarity of the filtrate.
Proximal tubule cells have Na\(^+/\)K\(^+-\)ATPases along their basolateral membrane which are active transporters that pump sodium out of the cells while driving potassium into the cells. This creates a negative membrane potential inside the cell and a Na\(^+\) gradient which stimulates the reabsorption of Na\(^+\) and Cl\(^-\) from apical transporters. Cl\(^-\) is released from the cell basolaterally through Cl\(^-\) carrying exchangers and cotransporters. This process is mostly responsible for the large amount, about 60-70\%, of NaCl and water that is reabsorbed by the proximal tubule\(^3^4\). The Na\(^+\) gradient is crucial for the cell because of the various Na\(^+\) dependent countertransporters and cotransporters that are responsible for the uptake of HCO\(_3^-\) and other solutes.

Another important function of the proximal tubule is the adsorption of metabolites from the glomerular filtrate. The human kidney filters about 180 g of glucose and 50 g of amino acids per day and 99.8\% of these metabolites are reabsorbed from the filtrate by the proximal tubules\(^3^4\). Glucose transport from the lumen through the proximal tubule cells is mediated by the two Na\(^+\)-dependent transporters SGLT2 and SGLT1. SGLT2 is expressed on the apical membrane of the S1 and S2 segments of the tubule and extracts the majority of the filtered glucose and functions as a cotransporter of glucose and one Na\(^+\) ion \(^2^1\). SGLT1 is located on the apical membrane of the S3 segment and cotransports two Na\(^+\) ions per glucose. The two transporters work effectively to reabsorb almost all of the filtered glucose from the glomerular filtrate. Glucose is then passively transported from the proximal tubule cells into the interstitial space through the Na\(^+\)-independent glucose transporters, GLUT2 and GLUT1.

The process of transporting amino acids is more complex with the transporters depending on the structure and ionic properties of the molecule. The majority of amino acids that are absorbed through the apical membrane are neutral amino acids transported by the Na\(^+\)-dependent cotransporter, B°AT1, which is located in the early portion of the proximal tubule\(^3^5\). Acidic amino acids are recovered through the Na\(^+\) and H\(^+\)-dependent cotransporter, while an antiporter governs the uptake of basic amino acids and cysteine, and exchanges them for a neutral amino acid\(^3^4,3^6\). This complex process leads to a majority of amino acids being reabsorbed and leaving only a small amount in the final urine\(^3^7\).

In addition to the absorption of molecules, the proximal tubules also play an important role in the excretion of organic anions and cations. The transporters involved are part of the SLC22 family of
transporters and are divided into groups based on substrates and mechanism, namely the organic cation transporters (OCTs), the organic anion transporters (OATs), and the zwitterion-cation transporters (OCTNs)\textsuperscript{21}. They transport organic and xenobiotic substrates such as drugs, toxins, hormones, pollutants, and cellular metabolites from the blood to the urine. There are three OCT transporters (OCT1, OCT2, and OCT3) and are located on the basolateral membrane (Fig. 2.1.4). They are potential-sensitive, reversible, and Na\textsuperscript{+}-independent, and transport organic cations and weak bases\textsuperscript{21}. In contrast, there are six OAT transporters which play a crucial role in the excretion and detoxification of drugs, toxins, hormones, and neurotransmitter metabolites. OAT1 is expressed on the basolateral membrane of the proximal tubule and functions as an organic anion-dicarboxylate exchanger by releasing \(\alpha\)-ketoglutarate or glutarate to provide the force for organic anion uptake into the cell. OAT2 is expressed in the liver and to a smaller degree in the kidney, where it is located on the basolateral membrane of the proximal tubule. OAT2 is Na\textsuperscript{+}-independent and an organic anion-dimethylodicarboxylate exchanger. OAT3 is an organic anion-dicarboxylate exchanger that is driven by the translocation of glutarate\textsuperscript{21}. It is also predominately located on the basolateral membrane of proximal tubule cells whereas OAT4 is located on the apical membrane. OAT4 plays a role in reabsorption of organic anions from the filtrate.

Additionally, another category of transporters localized to the proximal tubule is the ATP-binding cassette (ABC) transporter family. These include multidrug resistance proteins 2 and 4 (MRP2/4), breast cancer resistance protein (BCRP), and (P)-glycoprotein (MDR1/P-gp)\textsuperscript{20} (Fig. 2.1.4). The excretion of endogenous and xenobiotic anionic substances from the cell to the filtrate is performed by MRP2 and MRP4, which are localized on the apical membrane of the proximal tubule\textsuperscript{38}. These transporters are ATP-dependent pumps that modulate the movement of a broad range of substrates into the filtrate to be excreted from the urine.

*Ex vivo* studies have verified the transport of organic anions in the proximal tubule through the utilization of fluorescent dyes. For example, Maserceuw et al. isolated rat kidneys for *ex vivo* perfusion tests for the investigation of individual organic anion transporters. They supplemented perfusion media with the organic anion fluorescent dye, Lucifer yellow (LY). Results showed that LY was successfully cleared from the rat kidneys and this clearance could be reduced by the addition of the organic anion transporter inhibitor, probenecid\textsuperscript{39}. The LY clearance was also reduced by the addition
of the organic anion, α-ketoglutarate to the perfusion media, proving that LY is partially transported through the α-ketoglutarate dependent OAT transporters.

![Image of organic transporters in proximal tubule cells.](image)

**Figure 2.1.4. Schematic of the organic transporters in proximal tubule cells.** OATs are organic anion transporters and OCTs are organic cation transporters. Transporters of the SLC family are marked in blue and ABC transporters are marked in red. The movement of ions is shown with grey arrows. Adapted from Sánchez-Romero et al. Copyright permission granted by Elsevier Publishing Group.

To explore the organic transport process further, another experiment was performed using isolated killifish renal proximal tubules, which tubule ends reseal following isolation. LY was again added to the media and the tubule was imaged with confocal microscopy. Micrographs showed that the fluorescence intensity was the highest in the lumen of the tubule and the fluorescence intensity of the cells was higher than that of the medium, indicating that LY is transported through the cells and into the lumens of the tubule. By supplementing various inhibitors into the media and monitoring LY accumulation, Masereeuw et al. were able to show that proximal tubule cells uptake LY on the Na\(^+\)-dependent basolateral carrier for small organic anions, whereas about half of LY is transported to the lumen through the Na\(^+\)-independent transporters, potentially being the multidrug resistance-associated protein (MRP). This study demonstrates the complexity of organic transport through the proximal tubule and how xenobiotics can enter proximal tubule cells through one organic anion transport system and be secreted into the lumen on multiple carrier systems.
The proximal tubule has a complex role in the reabsorption of important nutrients and ions from the filtrate to maintain homeostasis and the transportation of potentially toxic compounds from the blood to the urine. In addition, the proximal tubule also secretes H\(^+\) and K\(^+\) ions to regulate the pH of the filtrate, has endocrine and immune characteristics, and performs hydroxylation of vitamin D. Altogether, the proximal tubule is one of the most important parts of the nephron and thus, functioning proximal tubules are essential to maintain healthy kidney function.

### 2.1.3 Renal tubulogenesis

In the previous section the many functions of the proximal tubule were described, highlighting their importance in the kidney. Due to the fact that they are the first tubule segment to be in contact with the filtrate, as well as being responsible for mass reabsorption of solutes and transportation of toxins, proximal tubules are the target of many renal pathologies and drug-induced toxicity. For these reasons, much interest is focused on the regeneration of proximal tubules, specifically on the generation of proximal tubules *in vitro*, where tubulogenesis can be thoroughly studied for regenerative therapy.

Renal tubulogenesis is a complex process involving the interplay of cell-cell interactions, extracellular matrix, and growth factors. The first known renal tubulogenesis model was established in 1991 by Montesano et al. with Madin-Darby canine kidney (MDCK) epithelial cells cocultured in collagen I gels with fibroblast or fibroblast conditioned medium\(^ {40} \). The study found that MDCK cells cultured in collagen I formed cysts, but when cultured in collagen I gel with fibroblast conditioned medium they underwent tubulogenesis. They also found that the pro-tubulogenic effect could not be repeated by the addition of epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), transforming growth factor beta 1 (TGF\(\beta_1\)), insulin-like growth factor I (IGF-1), insulin-like growth factor II (IGF-11), platelet derived growth factor (PDGF), or keratinocyte growth factor (KGF).

With further probing, the same lab discovered that hepatocyte growth factor (HGF) was indeed the fibroblast-derived molecule that induced tubulogenesis in MDCK cells\(^ {41} \). Hepatocyte growth factor, also known as scatter factor, was first identified as a regulator of liver regeneration. Since then, HGF was found to have stimulatory effects on a range of cell types, including the proximal tubule cells\(^ {42} \). HGF has also been discovered in the peritubular interstitium of the rat kidney, and results from *in
situ hybridization after induced injury in a rat kidney, suggest HGF was produced by endothelial cells. Montesano et al. proved the importance of HGF in MDCK tubulogenesis by incubating human fibroblast conditioned medium with HGF antibodies. This led to a dose-dependent inhibition of tubulogenesis with complete suppression at higher concentrations. It is also important to note that the medium used contained 5% fetal calf serum (FCS). FCS is a common supplement to cell culture media that contains a complex mixture of proteins, growth factors, hormones, nutrients, and trace elements. Nevertheless, this was a breakthrough in renal tubulogenesis at the time and many subsequent studies after have used this established model to study tubulogenesis.

Tubulogenesis mechanisms of epithelial cells such as mammary gland cells undergo morphogenesis through one of the following methods: rubber-sheet deformation or dissociation-reassociation (Fig. 2.1.5). Rubber-sheet deformation is when cells remain intact in their correct apical basolateral orientation and remodel through invagination or evagination. In the second process, dissociation-reassociation, some cells in the epithelium lose cell-cell contact, migrate, and the reassociate into a tubule. This second process is seen in nephrogenesis during kidney development.

Pollack et al. used the HGF-induced MDCK tubulogenesis model to investigate the mechanism behind renal tubule formation. Using basolateral and apical markers, they were able to follow the polarity of the epithelial cells throughout tubule formation. They found that cell-cell contacts are retained throughout all stages of tubulogenesis, yet polarity is transiently lost and then restored during the formation of tubule lumens in the regions of developing tubules. This process is shown in Figure 2.1.6 and can be divided into four morphologically distinct stages after the formation of cysts. First, individual cells from the cyst put out membrane protrusions into the extracellular matrix, this phase is called extensions. The extension is followed by chains of cells that are connected to the cyst. A few days later, cords forms that are two to three cells thick and have discontinuous lumens. During the fourth and final stage, the discontinuous lumens coalesce, become continuous with the lumen of the cyst, and form tubules.
Interestingly, the proposed method does not fall into the previously established mechanisms of rubber-sheet deformation or dissociation-reassociation, but instead has unique characteristics resembling parts from each process. For example, in the rubber-sheet deformation process, the cells keep their polarity, however MDCK cells were seen to transiently lose their polarity through the morphogenesis process (Fig. 2.1.6 B), yet they also did not detach from the cysts as the dissociation-reassociation process predicts. Therefore, MDCK cells undergo a novel process of tubulogenesis. It is also important to note that MDCK cells used in this study were derived from a heterogeneous parent line of MDCK cells, which are believed to be derived from the distal tubule or collecting duct of the nephron. Therefore, it is possible that MDCK cells have different mechanisms of tubulogenesis than proximal tubule cells, the subject of this thesis.

There also may be differences between tubulogenic stimulators. For example, Barros et al. found that the murine inner medullary collecting duct cell line (mIMCD-3) can form tubule structures when cultured in collagen I gels in the presence of serum whereas MDCK cells cultured in the same conditions were unable to form tubule structures. Nevertheless, understanding tubulogenesis mechanisms in one renal epithelial cell type can provide important insights into the modulators of tubulogenesis in another type. The following section will cover the extracellular matrix (ECM), growth factors, and proteases that have been found to play a role in tubulogenesis of renal epithelial cells, with emphasis on the renal tubule.
The extracellular matrix is an important factor in regulating tubulogenesis, as it provides mechanical stability, as well as adhesion sights for cells. Extracellular matrices also contain proteoglycans attached to glycosaminoglycan (GAG) chains that have the ability to bind to many growth factors through electrostatic charges. Therefore, extracellular matrix composition plays a vital role in contributing to epithelial morphogenesis. Santos et al. used the same conditions as the previously established HGF induced MDCK tubulogenesis model, except embedded in Matrigel instead of collagen I. Interestingly, MDCK cells did not form branching structures when cultured in Matrigel. Matrigel is a commercially available reconstituted basement membrane derived from mouse sarcoma tumors. It contains ECM components that are native to the renal basement membrane would be expected to mimic a more in vivo like environment compared to collagen I which is not a component of the renal tubule basement membrane (discussed in detail in Section 2.1.2). Even when the HGF concentration was increased fivefold compared to the 20 ng/ml HGF used for collagen gels, MDCK cells still did not form tubule structures, signifying the importance of ECM in tubulogenesis.
To further probe the effect of specific ECM components and determine stimulatory and inhibitory factors, Santos et al. mixed single extracellular components with collagen I and embedded MDCK cells in the resulting matrix. Specifically, the ECM components tested were laminin, entactin, collagen type IV, fibronectin, vitronectin, and heparan sulfate. Interestingly, the addition of laminin, entactin, and fibronectin to collagen I gels led to an increase in the amount of MDCK colonies with extensions compared to collagen I gels. In contrast, MDCK cells in collagen I mixed with heparan sulfate or collagen IV inhibited the number of colonies with extensions and resulted in round cysts. Vitronectin also inhibited the amount of processes and tubules formed in this condition showed a minimal amount of branching. Taken together, these results demonstrate that ECM composition can modulate tubulogenesis and drive the branching of renal structures.

The ability of the cells to degrade ECM is another important aspect of tubulogenesis, as cells need to be able to migrate and remodel the matrix to form tubule structures. Therefore, it is reasonable to assume that matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) also play an important role in tubulogenesis. HGF has been seen to increase the release of certain MMPs from MDCK cells and proximal tubular epithelial cells. Kadono et al. found that membrane type-1 MMP (MT1-MMP) mRNA was upregulated in HGF-treated MDCK cells grown in collagen gels. When the cells were transfected with MT1-MMP antisense RNA to repress translation of MT1-MMP, tubule formation was inhibited. Another way they could reduce tubule formation was with the addition of the broad spectrum MMP inhibitor, BB-94, or the intrinsic tissue inhibitor of metalloproteinase-2 (TIMP-2). However, TIMP-1 did not have the same effect.

Interestingly, a decade later, Hellman et al. found that TIMP-1 is highly upregulated in MDCK cells treated with HGF in a collagen matrix. It is possible that TIMP-1 is a promoter rather than an inhibitor of tubulogenesis, a potential reason for this is that TIMP-1 induces the upregulation of MT1-MMP, which we previously have stated is involved in tubulogenesis. Another reason is that TIMP-1 has anti-apoptotic function of TIMP-1 reported in other studies which could help regulate cell apoptosis and create the lumens necessary for tubulogenesis. The same study also found that MMP-13 mRNA was also highly upregulated with HGF treatment. Using MMP13 knockdown cells, they revealed that MMP13 is necessary for the redifferentiation stage of tubulogenesis. Therefore, both MMPs and TIMPs participate in regulating tubulogenesis.
Another factor and the most researched to date, is the ability of soluble cues to induce tubulogenesis. It has already been stated that HGF is necessary for MDCK tubulogenesis. However, in these studies medium is supplemented with FBS which contain a variety of growth factors and thus, a combination of growth factors might be necessary to induce tubulogenesis. Importantly, the exact combination of factors that is responsible for proximal tubulogenesis is still unknown, as well as the regulatory mechanism behind ECM driven tubulogenesis. Results from several studies that investigated the effect of growth factors will be summarized with a focus on proximal and distal tubule cells, since they are the most relevant to this thesis.

Similar to MDCK cells, human primary renal proximal tubular epithelial cells (RPTEC) form tubular structures in the presence of 20 ng/ml of HGF when cultured in collagen/Matrigel. However, without HGF, the cells remained rounded after 5 days. This was also seen when the medium was supplemented with 250 ng/ml of BMP-7, EGF, IGF-1, or follistatin, suggesting that these proteins do not play an active role in proximal tubulogenesis\(^57\). The HGF induced tubulogenesis could be significantly increased by culturing the RPTEC containing gels in transwells with human umbilical vein (HUVEC) endothelial cells seeded on the bottom of the plate. The same effect was also seen when HUVEC conditioned medium was used instead of the co-culture system. The increase in tubulogenesis could not be mimicked by the addition of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF-2), or platelet-derived growth factor (PDGF). The HUVEC conditioned medium was further analyzed for the presence of MMPs and TIMPs. It was found that the conditioned medium contained MMP-1, TIMP-1, TIMP-2, and small amounts of MMP-2, MMP-3, and TIMP-4. When the conditioned medium was pretreated with recombinant TIMP-1 or TIMP-2 the enhanced tubulogenesis was reversed\(^57\).

Mouse proximal tubule cells incubated with 50 ng/ml VEGF underwent tubulogenesis similar to HGF-treated samples\(^58\). Interestingly, they also showed that specific isoforms were involved. VEGF-165 induced branching whereas VEGF-121 did not. This demonstrates that the mechanism behind tubulogenesis is highly sensitive and specific to growth factors. As the physiological environment is extremely complex, it is likely that the ideal condition to promote tubulogenesis involves a variety of growth factors. Bowes et al. found that when FGF-1, EGF, IGF-1, or FGF-7 was added to medium with HGF, branching and proliferation of primary rat proximal tubule cells was enhanced compared to cultures with HGF alone\(^59\). The optimal condition was the combination of HGF, FGF-1, EGF,
and IGF-1 that led to a higher proliferation and size of branched structures. Interestingly, they also discovered that the addition of the sulfated glycosaminoglycan, heparin, to the medium resulted in the maximal HGF-induced cell proliferation. This is most likely due to heparin’s ability to bind and stabilize growth factors and proteins. Overall, tubulogenesis is a complex process that can be modulated through extracellular matrix, proteases, growth factors, and additional factors not yet discovered. Further exploration into renal proximal tubulogenesis can lead to new important insights into the field of regenerative medicine. However, a tunable in vitro renal tubulogenesis model is necessary for the progress of such a study.

2.2 Drug-induced nephrotoxicity

More than 20 million people in the United States alone are estimated to have some degree of chronic kidney disease (CKD). CKD is a progressive loss of renal function that can lead to end stage renal disease (ESRD), and ultimately end in renal failure. It is estimated that 19-25% of all cases of severe acute renal failure in patients is due to drug-induced nephrotoxicity, highlighting the need for better preclinical assays. The kidney is highly susceptible to drug toxicity, owing to its anatomy and function. The kidney continuously receives a large volume of blood that exposes the tissue to many solutes and leads to high intracellular concentrations being reabsorbed, especially in the proximal tubules. The proximal tubules also uptake toxic agents from the peritubular capillaries and metabolize drugs, xenobiotics, and other substances into toxic metabolites and reactive oxygen species. This process results in elevated concentration of toxins in the tubule which can cause injury through direct toxicity or through ischemic damage from reactive oxygen species created as byproducts of metabolism. The glomerulus and the proximal tubule are the sites of more than 90% of drug related toxicity, as can be seen in Fig. 2.2.1B.

Current nephrotoxicity assays rely on 2D cell culture and animal models. However, cells grown on conventional 2D tissue culture substrates lack the cell-cell and cell-matrix interactions of the in vivo environment. For this reason, 2D cell cultures often require much higher doses of drugs to induce a toxic response compared to the human reaction to toxicity. They also have a limited growth area, making them unsuitable for long term studies.
In contrast, animal models provide a physiological 3D environment. However, studies have found that a limited number of animal experiments accurately predict the human response to drugs. As a result, less than 10% of newly developed drugs make it past clinical trials. Therefore, there is a critical need for more reliable kidney models to recapitulate the human environment. 3D cell culture
models are a promising alternative to conventional methods, as they can be used with human cells and better mimic the 3D human physiological conditions. They also allows for the analysis of biomarkers that have been found to indicate nephrotoxicity in vivo and the exploration of new biomarkers.

Biomarkers are important to predict toxicity in preclinical studies to prevent nephrotoxic drugs entering the market and to indicate injury in humans at an early stage. Commonly used biomarkers to detect kidney injury in preclinical or clinical studies are serum creatinine (SCr) and blood urea nitrogen (BUN). However, they both have several limitations. The concentration of both biomarkers is controlled by many factors, which can cause a delay in detection of injury and also does not pinpoint the specific renal injury and location. SCr also cannot detect histological injury in nephrotoxicity preclinical studies in animals and humans. BUN is a waste product from the metabolism of proteins. BUN levels can change based on the volume of the filtrate, exogenous protein loads such as protein supplementation, and endogenous catabolic states, which can all increase BUN levels without any kidney injury. Therefore, these biomarkers are not specific and sensitive enough for the early indication of nephrotoxicity in humans or the recognition of nephrotoxicity in preclinical trials.

For these reasons, scientists have recently been investigating novel biomarkers to detect human nephrotoxicity. A list of kidney injury biomarker candidates that have recently been investigated are shown in Fig. 2.2.1A along with the location of injury in the nephron that they detect. The noninvasive biomarker, kidney injury molecule (KIM-1) is of particular interest to this thesis because it is an early biomarker for proximal tubular damage. KIM-1 is a type 1 transmembrane protein that is upregulated in proximal tubule cells after injury. The ectodomain of the protein can be shed by proximal tubule cells and measured from the urine during the first 12 hours of tubular injury. KIM-1 is highly expressed in the tissue and/or urine of experimental and clinical studies in association with fibrosis, inflammation, and in ischemic acute renal failure. Therefore, KIM-1 may be a promising noninvasive biomarker for the early detection of proximal tubule injury.

Many drugs are toxic to the kidney, especially the proximal tubule (Fig. 2.2.1B). Even toxins affecting other regions of the nephron, can still pose problems on the proximal tubules. One drug that is particularly toxic is the highly potent chemotherapy drug cisplatin.
diaminedichloro platinum(II)). Cisplatin is commonly used for the treatment of testicular, head, neck, ovarian, cervical, genitourinary tract, lungs, and breast, as well as other types of cancer\textsuperscript{73}. Cisplatin is a platinum-based simple inorganic molecule that is believed to bind to DNA, forming crosslinks that create defective DNA templates\textsuperscript{74}. This reaction stops DNA synthesis and replication. In cancer cells which are rapidly dividing this can induce DNA damage leading to cell death. Although cisplatin has been found to be a successful cancer treatment, its dosage and use is limited based on its cumulative nephrotoxicity and neurotoxicity\textsuperscript{75}. Acute kidney injury (AKI) occurs in 20-30\% of patients treated with cisplatin\textsuperscript{76} and can cause other renal complications such as hypomagnesemia, fanconi-like syndrome, distal renal tubular acidosis, hypocalcemia, renal salt wasting, renal concentrating defect, hyperuricemia, transient proteinuria, erythropoietin deficiency, thrombotic microangiopathy, and chronic renal failure\textsuperscript{76}. There is a higher risk of nephrotoxicity in cisplatin-treated patients with higher doses and frequency and have the following characteristics: older age, female, smoker, hypoalbuminemia, and pre-existing renal insufficiency\textsuperscript{76}.

Cisplatin is particularly toxic to the renal proximal tubules, which accumulates a concentration of cisplatin that is 5 times more than the serum concentration, which contributes to its toxicity in the cells\textsuperscript{77,78}. Two membrane transporters have been identified as responsible for the uptake of cisplatin in proximal tubule cells, Ctr1 and OCT2. Ctr1 is a high affinity copper transporter that is localized to the basolateral membrane of the proximal tubule and is also responsible for the partial uptake into cancer cell lines\textsuperscript{74}. OCT2 is an organic cation transporter that is also located on the basolateral membrane of the proximal tubule cell and plays a role in cellular uptake of cisplatin\textsuperscript{74}. Overexpression of OCT2 in human proximal tubular cells resulted in increased uptake and sensitivity to cisplatin and the OCT2 inhibitor, cimetidine, has been shown to decrease cisplatin uptake in renal tubular cells\textsuperscript{79}.

The mechanism of cisplatin induced nephrotoxicity is a complicated process that involves multiple pathways. \textit{In vitro}, it was found that low doses of cisplatin induces apoptosis in proximal tubular cells whereas high concentrations led to necrosis\textsuperscript{80}. However, nephrotoxic doses \textit{in vivo} lead to an increased level of both apoptosis and necrosis in the kidney\textsuperscript{76}. Once in the cell, cisplatin undergoes metabolic activation into more potent toxins. While still in the blood circulation, cisplatin forms glutathione conjugates. They are then cleaved to cysteiny1-glycine-conjugates by gamma glutamyl transpeptidase (GGT) expressed on the surface of proximal tubule cells and then subsequently,
metabolized to cysteine-conjugates by aminodipeptidases on the proximal tubule cells. These cysteine-conjugates then enter into the proximal tubule cells and are further metabolized by cysteine-S-conjugate beta-lyase to highly reactive thiols. This reaction then causes injury in the tubular and oxidative stress as a result from the reaction, as a result inflammation ensues, followed by vascular injury in the kidney. Understanding the pathway of cisplatin-induced nephrotoxicity is important for identification therapeutic treatments to inhibit its nephrotoxic effects, potentially through inhibiting one of the crucial stages of cisplatin metabolism. 3D renal models offer a promising way to study this mechanism in vitro where the effects can be easily monitored. The next section will cover the current state of biomaterial-based kidney models.

2.3 3D in vitro biomaterial-based renal models

As briefly mentioned in earlier sections, there is a critical need for more reliable and representative human renal models that can be used for the study of renal tubulogenesis, as well as nephrotoxicity. The ideal model would recapitulate the human 3D structure and function of proximal tubules in a robust matrix that can be easily analyzed in throughput screenings for toxicity or regenerative medicine applications. There are several approaches to obtaining the ideal 3D renal model including kidney organoids from stem cells, natural and synthetic hydrogels, kidney-on-a-chip, 3D printing, and decellularized scaffolds. Keeping with the concept of the thesis, this section will focus on the advantages and disadvantages of kidney organoids and tubule models in natural and synthetic biomaterials.

Recent progress in the generation of kidney organoids from human stem cells can be considered highly promising for creating more realistic human in vitro models. Organoids utilizing induced pluripotent stem cells have great potential in personalized medicine because they can easily be obtained compared to primary renal cells that can only be acquired invasively. Freedman et al. formed tubular organoids by culturing human pluripotent stem cells in between two layers of Matrigel. The cells formed cavitated spheroids in the Matrigel and after a change of media and addition of glycogen synthase kinase-3β (GSK3β) inhibitor (Chiron Technologies 99021 - CHIR99021) and B27 supplement, the spheroids disaggregated and formed tubular organoids. The role of Matrigel is unclear in the system and was not thoroughly investigated.
The report by Freedman et al. showed very exciting data for the use of stem cells in creating renal organoids for toxicity and disease modeling using CRISPR/Cas9 gene editing\textsuperscript{84}. However, the tubular structures that formed were much larger than human proximal tubules \textit{in vivo} and the culture also contained a distribution of non-renal cells, which can potentially disrupt results. Interestingly, in the reported manuscript, they compared induced pluripotent stem cells with embryonic stem cells and found that induced pluripotent stem cells led to a lower differentiation efficiency into tubular organoids than human embryonic stem cells\textsuperscript{84}. A possible reason for this is that induced pluripotent stem cells can retain epigenetic characteristics of their somatic tissue of origin\textsuperscript{86}. Although embryonic stem cells have high differentiation purity, 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 \textit{in vivo} \textsuperscript{84,87,88}. Also, the use of stem cells in a culture model adds additional differentiation steps that prolongs the culture time. Another important factor for the study of renal regenerations is that stem cells, especially embryonic stem cells, recapitulate the developmental kidney nephrogenesis rather than portraying how adult renal cells respond to stress and stimuli. For these reasons, the application of primary renal tubule cells for renal models is advantageous for models of adult nephrotoxicity and renal regeneration.

An alternative to organoid models, are tubule/tubulogenesis models with differentiated renal cells that offer a more simple and straightforward culture. Previously described 3D renal tubulogenesis models use reconstituted collagen I, Matrigel\textsuperscript{TM}, or a combination of these materials\textsuperscript{40,41,49,57,59,89–93}. The first renal tubulogenesis model was reported in 1991 which established a tubulogenesis model using MDCK cells resuspended in collagen I matrix with the addition of fibroblast conditioned medium or HGF to the culture\textsuperscript{40,41}. Since then, this model has been extensively investigated and revealed important factors that contribute to renal tubulogenesis, which is described in Section 2.1.3. While this method was the gold standard for many years, it is not the ideal model to study human proximal tubulogenesis. This is because of two main factors: 1) MDCK cells are from canine which can vary to human cells and 2) MDCK are derived from the distal tubule or collecting duct of the nephron and therefore, have different functions and potentially different tubulogenic mechanisms than proximal tubules.

To create a model that is more representative of proximal tubules, some studies have used primary proximal tubule cells or a heterogeneous population of human renal cells embedded in collagen I or
a mixture of collagen I and Matrigel\textsuperscript{57,94,95}. While these matrices support the growth of renal tubular cells, the formed tubular structures do not accurately portray the size and/or functions of the human proximal tubule. They generate tubule-like structures that are orders of magnitude smaller than \textit{in vivo} human proximal tubules and also offer limited evidence of a clear lumen\textsuperscript{57}. Similar to the MDCK model, the primary proximal tubule models often require the addition of HGF, EGF, or FBS to the medium to stimulate tubulogenesis\textsuperscript{57,59,95,96}.

DesRochers et al. embedded immortalized human renal cortical cells in a matrix made of a 50:50 mixture of Matrigel and collagen I. The mixed population of renal cells formed small tubule-like structures after 2 weeks in culture in the Matrigel/collagen mixture. The model was used to systematically compare nephrotoxicity outcomes in 3D versus 2D cell culture\textsuperscript{89,97}. 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 \textit{in vivo} environment. However, even after 4 and 8 weeks of culture, the branched structures remained small, with only a 1 or 2 cell diameter and there was no evidence of a lumen shown\textsuperscript{89}.

One of the disadvantages of using naturally derived biomaterials is that they often have batch-to-batch variations which inhibit the reproducibility of the cultures. They are also difficult to handle, requiring cold temperatures and adjustment of pH(with collagen gels) that make them suboptimal for high throughput screening. Moreover, these materials lack tunability in mechanical and biofunctional properties, which is required for testing the impact of exogenous signals on renal tubulogenesis.

Synthetic matrices could circumvent the problems of naturally derived matrices by offering controlled, tunable material characteristics that can be customized to stimulate renal tubulogenesis. To date, the only synthetic hydrogel used as a renal model was reported in 2012 by Astashkina et al. and was made with hyaluronic acid(HA) and poly(ethylene glycol) diacrylate (PEGDA)\textsuperscript{98,99}. This report showed that synthetic hydrogels have the potential to maintain murine tubule tissue for long term culture. Proximal tubule fragments were digested from murine kidneys and embedded in the HA-PEGDA hydrogel. The murine tubules retained function (cathepsin B, alkaline phosphatase, and gamma glutamyltransferase) when cultured in the hydrogels. The model was also tested for application as a nephrotoxicity assay by incubating the hydrogel samples with cisplatin, doxorubicin, and 4-aminophenol, which resulted in increased levels of inflammatory cytokine release.
This HA-PEGDA model was a huge development in the field by showing the many functional tests and applications that can be utilized with 3D renal models. However, the model is not clinically relevant because proximal tubules cannot be extracted from living humans and results gathered from mouse tubules can contain interspecies differences. In addition, the model does not provide a model for tubulogenesis and the growth of structures was not shown. Therefore, it has limited use for investigating pro-tubulogenic factors for regenerative medicine.

Although these studies have been important breakthroughs in the field of 3D models, the optimal renal tubulogenesis model has not yet been established. The ideal renal tubulogenesis model would accurately recapitulate the structure, size, and function of the proximal tubules. The model should employ a tunable matrix where physical and biomolecular parameters can be independently investigated to allow for investigating renal regenerative therapies and disease modeling. A renal tubulogenesis model that functions similar to in vivo human proximal tubules could also be used as a reliable nephrotoxicity model, limiting the need for animal models. Work presented in this thesis will show the use of in situ forming starPEG-heparin hydrogels as a renal tubulogenesis model. A summary of the characteristics of starPEG-heparin hydrogels will be described in the following section.

2.4 In situ forming starPEG-heparin hydrogels

In this thesis, biohybrid hydrogels were used to create an in vitro 3D renal tubulogenesis model. The hydrogels are composed of two components, the synthetic, flexible polymer, four-armed poly(ethylene glycol) (starPEG) and the glycosaminoglycan, heparin. This hydrogel platform has been successfully employed as a cell culture model for a variety of cell types\textsuperscript{100-103}. PEG is a hydrophilic, hydrolytically non-degradable, non-toxic polymer that is highly soluble in water, making it useful for various biomaterial applications. Additionally, PEG undergoes only limited metabolism in the body and whole polymer chains can be eliminated either through the kidneys by glomerular filtration if their molecular mass is less than 30 kDa and through the liver when molecular mass is greater than 30 kDa\textsuperscript{104}. Also, the terminal groups of PEG can be easily modified with functional groups making it a versatile building block for biomaterials.

The other main component of the hydrogel network is heparin, a glycosaminoglycan composed of repeating disaccharide units containing iduronic acid and glucosamine. Heparin is only found in small
amounts in the human body\textsuperscript{105}. However, it has a similar structure to heparan sulfate which is endogenously located on the surfaces of mammalian cells and a common component of the extracellular matrix\textsuperscript{60}. Heparan sulfate contains less sulfation groups than heparin. However, heparin is more often used for biomaterial applications because it is readily available.

Due to its high content of sulfation groups, heparin has the highest negative charge of any biological macromolecule\textsuperscript{60}. The high negative charge allows it to electrostatically bind and stabilize numerous positively charged growth factors and proteins. Heparin also contains adhesion sites for a variety of growth factors such as HGF, HB-EGF, and FGF-2. Moreover, heparin has the ability to bind to extracellular matrix (ECM) molecules, such as collagen IV, one of the main components of the renal basement membrane\textsuperscript{50,106,107}. Altogether, using heparin as a building block in hydrogels can create a more \textit{in vivo}-like environment for cell culture.

To create three-dimensional (3D) cultures for embedding cells, \textit{in situ} forming biohybrid hydrogels were formed by covalently crosslinking starPEG and heparin through Michael-type addition. This reaction is beneficial because it has no side products, does not require a catalyst, and takes place quickly under physiological conditions\textsuperscript{108,109}. Heparin is first functionalized with maleimide for such a reaction. The carboxylic groups on the heparin are activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfo-succinimide (sNHS) which creates a sNHS-heparin ester. This then reacts with the amine group on the N-(2-aminoethyl)maleimide which forms heparin-maleimide conjugates (Fig. 2.3.1 top panel). Using this method, up to 8 maleimide groups can be attached to the back bone of heparin. Commercially available thiol-end-functionalized starPEG is then mixed with the maleimide-functionalized heparin, where the Michael-type addition reaction takes place. Specifically, acting as a nucleophile, the thiol group of the starPEG shares electrons with the carbon-carbon double bond of the maleimide group on the heparin. This creates a covalent bond between the starPEG and heparin and allows for the formation of a crosslinked network (i.e. hydrogel)(Fig. 2.3.1 bottom panel)\textsuperscript{110}. 
Figure 2.3.1. Schematic describing starPEG-heparin hydrogel synthesis. Top panel shows the functionalization steps of heparin to form heparin-maleimide conjugates. Middle panel how starPEG can be functionalized with ECM peptides (SIKVAVGWCG) and MMP-cleavable peptides (GCGPQGIWGQGCQ). Adapted from Tsurkan et al. Copyright permission granted by John Wiley and Sons Publishing Group.

A key advantage of starPEG-heparin hydrogels over other hydrogel systems used as cell scaffolds is its ability to bind and release growth factors and cytokines. Another advantage of the hydrogel is the ability to decouple the physical and biomolecular characteristics to investigate their individual effects on cell behavior. The crosslinking density of the hydrogel and consequently, the bulk mechanical properties, can be modulated by easily adjusting the molar ratio of starPEG to heparin (γ). By keeping the heparin concentration constant, the mechanical properties can be tuned independently of the biomolecular properties of the hydrogel. The maleimide on the heparin can be further functionalized with cell-adhesion sites such as Arg-Gly-Asp (RGD) containing peptides to further replicate the in vivo environment and promote cell adhesion. To further imitate the extracellular matrix, the starPEG component can be functionalized with ECM peptides like laminin or collagen (Fig. 2.3.1 middle panel). A MMP cleavable peptide sequence can also be attached to
starPEG to allow cells to degrade and remodel the matrix, similar to how they would in vivo (Fig. 2.3.1).

As already alluded to, heparin makes starPEG-heparin hydrogels advantageous due to its ability to modulate growth factor and cytokine sequestration, stabilization, protection, and sustainable release. Several studies have shown the successful adhesion and release of growth factors. Relating to the scope of this thesis, heparin has been previously reported to govern the release and bioactivity of growth factors (FGF-2) from proximal tubule epithelial cells and decrease fibrosis and inflammation in models of renal disease. The binding and release of growth factors and cytokines can be further modulated by the removal of sulfation groups on the heparin.

Overall, starPEG-heparin hydrogels offer a highly tunable system that is ideal for probing cell behavior in in vitro cell models. This hydrogel platform has already been shown to be successful as an in vitro breast cancer model, a mammary epithelial model, and an angiogenesis model. The broad range of modulation allows the hydrogels to be further functionalized to continuously explore a single cell type or population of cells. For these reasons, starPEG-heparin hydrogels could have great potential as a renal tubulogenesis model. With this biohybrid hydrogel platform, mechanics, growth factor/cytokine presentation, ECM peptides, and degradability can be independently investigated to determine their effect on renal tubulogenesis. These results could provide important insights for the field of renal regenerative medicine. In addition, tubule structures generated in starPEG-heparin hydrogels could be used for nephrotoxicity assays and disease modeling.
Chapter 3

Materials and Methods

3.1 Preparation of hydrogel networks

For cell experiments, 25 μl hydrogel drops were formed on a hydrophobic microscope slide coated with Sigmacote® (Sigma-Aldrich, Munchen, Germany). After polymerization, the hydrogel was moved to a 24 well plate containing 1 ml of medium. Gel discs were used for rheology by forming 67 μl gels in between two 9 mm Sigmacoted coverslips. After polymerization, both cover slips were removed and the resulting hydrogel discs were swollen in cell culture medium. All phosphate buffered saline (PBS) used for hydrogel preparation was supplemented with 1% penicillin/streptomycin (P/S). For growth factor binding studies, 25 μl hydrogels were formed in low protein binding eppendorfs (Protein LoBind Tube, Eppendorf AG, Germany). All materials used and methods for peptide and conjugate synthesis can be found in the Appendix (Chapter 6).

3.1.1 Formation of starPEG-heparin hydrogels

Hydrogels were fabricated as previously described. All hydrogel material was dissolved in phosphate buffered saline (PBS) with 1% P/S. In brief, heparin-maleimide (MW = 10,000) was dissolved in ¼ of the total gel volume. Proximal tubule cells were then added to the heparin-maleimide solution in ¼ of the total gel volume to make 2 x 10^6 cells/ml (50,000 cells/gel). Thiol end-functionalized four arm starPEG (MW=10,000, JenKem Technology, China) or starPEG-MMP was dissolved in ½ of the total gel volume. The cell/heparin solution was then mixed with the starPEG/starPEG-MMP solution to form a 25 μl hydrogel. Gelation time of the hydrogels was ~30 seconds. For all cell experiments, a 25 μl gel was cast onto a microscope slide coated with Sigmacote® (Sigma) for 20 min at room temperature. After polymerization, the gels were then moved to a 24 well plate with warm media. The stiffness of the hydrogel was modulated by varying the molar ratio (γ) of starPEG/starPEG-MMP to heparin from 0.63 to 1.5. Synthesis of starPEG-MMP and heparin-maleimide conjugates can be found in the Appendix (Chapter 6).
3.1.2 Formation of starPEG-MMP-starPEG hydrogels

Degradable starPEG-MMP-starPEG gels were formed in a similar manner as starPEG-heparin hydrogels. Specifically, starPEG-MMP was dissolved in ¼ of the total gel volume and then mixed with the cells. StarPEG-end terminated with a maleimide group (starPEG-mal) was dissolved in ½ of the total gel volume and subsequently the pH was adjusted to 4-5. The solution was then mixed with the starPEG-MMP/cell mixture to form the hydrogels.

3.1.3 Formation of Matrigel samples

For Matrigel samples, cells were centrifuged, resuspended with Matrigel™ (BD Science), and then cast on microscope slide at 50 μl/gel. A 50 μl Matrigel volume was used because of its comparable size to starPEG-heparin hydrogels after swelling. Cell embedded-Matrigel was then incubated at 37°C for 20 min and then transferred to warm media. Each Matrigel gel contained 50,000 cells.

3.1.4 Rheometric measurement of hydrogel discs

Hydrogels were swollen in cell culture media overnight at 37°C. Oscillating rheology measurements were then performed on a rotational parallel plate rheometer (ARES LN2, TA Instruments, Germany) at 25°C, as described previously. Frequency sweeps were performed with a shear frequency range of $10^1 – 10^2$ rad s$^{-1}$ at 10% strain. The mean storage modulus was then calculated.

3.1.5 HGF functionalization of starPEG-heparin hydrogels

StarPEG-MMP-heparin hydrogels were functionalized with HGF using standard hydrogel preparation protocols followed in Section 3.1.2. Recombinant human HGF (Peprotech, Germany) was diluted to 100 μg/ml. During gel formation, HGF was added to the heparin-maleimide in 1/8 of the total gel volume. For the 25 μl gels used in these studies, heparin-maleimide was first dissolved in PBS in 1/8 of the total gel volume. The heparin-maleimide solution was then mixed with HGF diluted to 1/8 of the total gel volume by pipetting, resulting in ¼ of the total gel volume. During this time HGF can bind to heparin-maleimide through known binding sites and/or electrostatic interactions. The HGF-heparin solution was then mixed by pipet with HK-2 cells that were resuspended in ¼ of the total gel volume. 14 μl of the resulting solution was then mixed with 14 μl
of dissolved starPEG-MMP to form 25 μl gels. The hydrogels were cast on sigmacoted slides and then transferred to wells after polymerization.

### 3.1.6 Binding of HGF in HCS to hydrogels using Multiplex assay

To identify the amount of HGF in blood serum and how it binds to hydrogels, human umbilical cord serum was incubated with the hydrogels. Due to the lack of bovine antibodies, human umbilical cord serum (HCS; Human cord serum pooled, Lee Biosolutions, Inc., USA) was used as an alternative serum source. HGF was measured in 100% serum or 10% serum diluted in DMEM medium to stimulate cell culture experiments. Solutions were made in low protein binding eppendorfs or centrifuge tubes coated with bovine serum albumin to prevent unspecific protein binding. StarPEG-heparin hydrogels with 18 μl volume were formed at the bottom of 0.5 ml protein LoBind eppendorf tube (Eppendorf AG). Two hydrogel crosslinking densities were used 0.63 and 1.0, referred to as gel 1 and gel 2, respectively in the results. Collagen gels were also formed as a control at the following a concentration of 0.9 mg/ml and 3 mg/ml referred as collagen 1 and collagen 2, respectively. An additional control was made with soluble unfunctionalized heparin. For this control, 0.55 mg heparin, which is the amount in each starPEG-heparin hydrogel, was added to a 0.5 ml protein low bind eppendorf tube and mixed with the respective solution by pipetting. For immobilization of the analyte to the material, 300 μl of HCS containing solution was incubated with the hydrogel material, soluble heparin, or alone for 24 hours at room temperature. After the incubation, the solution was removed and immediately frozen at -80°C for later analysis. For cytokine quantification, samples were thawed and analyzed for HGF concentration using a human cancer 8-Plex-kit (Bio-Rad) measured with the Bio-Plex 200 System (Bio-Rad) according to the manufacturer’s instructions.

### 3.2 Culture of proximal tubule epithelial cells

#### 3.2.1 Cultivation of human proximal tubule cell lines

HK-2 human kidney proximal tubule epithelial cells (ATCC CRL-2190) 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 (Radboud university medical center,
Nijmegen, 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\textsuperscript{12}. 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.

3.2.2 Cultivation of primary human proximal tubule cells

Primary human renal proximal tubule epithelial cells were purchased from American Type Culture Collection (ATCC, #PCS-400-010). Primary tubule cells were cultured in the same media as ciPTEC, but supplemented with 0.5% FBS instead of 10%. Primary tubule cells were used for experiments at passages 2-4. Culture medium was changed every 2-3 days for all cell experiments.

3.3. In vitro assays

3.3.1 Tubulogenesis quantification

Three phase contrast images were taken from each hydrogel, weekly for 4 weeks (n=3 hydrogels/condition). At least 80 colonies from each gel were manually counted and categorized according to their stage of tubulogenesis which was categorized as round spheroids, spheroids with processes (“spiny spheroids”), or tubular structures. The values of all gels in the experiment were summed together and percent of tubulogenesis was quantified. The experiments were then averaged together which is shown in the presented graphs. Experiments were performed in triplicate and differences between experiments are shown with standard deviation. Colonies were considered to be tubules when their length to diameter ratio was greater than 5. Tubulogenesis results are expressed as percentage of total colonies counted.

3.3.2 Shape and size analysis of renal structures

After 4 weeks in culture, phase contrast images were acquired and area, perimeter, and circularity of colonies were quantified using Image J software (NIH, USA). Circularity measurement determines the roundness of an object where a value of 1.0 indicates a perfect circle and a value approaching 0.0
indicates an elongated structure. Three phase contrast images were acquired from each hydrogel and three hydrogels were made per condition. For starPEG-heparin and starPEG-MMP-starPEG samples, 20 structures per image were analyzed and for starPEG-MMP-heparin hydrogels, 10 structures per image were analyzed. This is due to the difference in structure size, in starPEG-MMP-heparin hydrogels, the large structures resulted in an average of 10 colonies per image. Colonies from one experiment were pooled together and summed for the area or perimeter analysis and averaged in the case of the circularity measurement. The total value for the experiment was then averaged with the totals from other experiments to get the value in the presented graphs. The difference between experiments is shown with standard deviation. Experiments performed in triplicate.

3.3.3 Metabolic activity assay

Metabolic activity was assessed by PrestoBlue assay. A 1:10 dilution of PrestoBlue Reagent (ThermoFisher Scientific) in warm media was added to each sample. Samples were incubated at 37°C for 2 hours. Then 100 μl of solution was transferred in duplicate into a 96 well plate for detection. Fluorescence was measured using a Tecan Genios plate reader (Tecan Deutschland GmbH) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. When comparing different matrices, hydrogels in each condition were first moved to a new 24 well plate to eliminate effects of cells outside of the hydrogels.

3.3.4 Cytotoxicity assay (LDH release)

LDH secretion was measured in cell culture supernatant using the LDH cytotoxicity assay (Takara, USA), following the manufacturer's instructions. Supernatant was taken from each sample in replicates of 3. Reaction mixture was incubated with supernatant for 15-20 minutes at room temperature and then absorbance was measured using a Tecan Genios plate reader (Tecan Deutschland GmbH). The positive control group was treated with 1% Triton X-100 to measure the maximum LDH release from 100% dead cells.

3.3.5 Organic anion transport assay

Transepithelial transport was analyzed by incubating the hydrogel cultures with the fluorescent organic anion, Lucifer Yellow(LY). After 4 weeks in culture, media was changed to 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 0.5% FBS. Samples were imaged with confocal and then Lucifer Yellow CH dilithium salt (Sigma, 100 μM) was added to media and incubated with the hydrogels for 1h. For washout experiments, media with LY dye solution was removed, replaced with dye free media, incubated for 20h, and then imaged with confocal microscopy.

### 3.3.6 Nephrotoxicity Assay

After 4 weeks in culture, media was removed on hydrogel samples and replaced with serum-free media containing 0 to 100 μM cis-Diammineplatinum (II) dichloride (cisplatin, Sigma) for 48 hours. A 2 mM stock solution of cisplatin was made by dissolving 6 mg of cisplatin in 10 ml of MilliQ water. The stock solution was vortexed and sonicated for 10-20 minutes until the majority of the powder was dissolved. The stock solution was then diluted further in MilliQ water to make solutions of 0.5 mM, 1.0 mM, 1.5 mM, and 2 mM. Finally, 50 μl of each cisplatin solution was added to a single well of a 24 well plate containing 950 μl of serum free DMEM/F-12 medium, making a final concentration of 25, 50, 75, and 100 μM. MilliQ water was added to control wells at the same volume. After incubation with the drug, metabolic activity and cytotoxicity were measured (Metabolic activity and cytotoxicity assays described in sections 3.3.3 and 3.3.4, respectively). Expression of the renal injury biomarker, KIM-1, and apoptotic cells were analyzed through immunofluorescence.

### 3.4. Microscopy techniques

#### 3.4.1. Phase contrast microscopy

For standard analysis of cell culture and image acquisition for the quantification of tubulogenesis and tubular structures, a Leica inverted microscope (Leica DMIL, Leica Microsystems, Germany) with Leica application software (LAS software, Leica Microsystems, Germany) was used. All non-fluorescent images of renal structures are greyscale phase contrast micrographs.

#### 3.4.2. Fluorescent staining

Samples were fixed in 4% performaldehyde for 20 min. Samples were washed 2 times with PBS and then immunostained immediately or embedded in OCT solution and snap-frozen on dry ice for
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cryosectioning. Samples were blocked with 5-10% goat or donkey serum in PBS or PBS with 0.5% Triton X-100 for 1 hour and then incubated overnight with primary antibodies at 4°C. After 3 washes with PBS/0.1% Triton X/1% donkey or goat serum, secondary antibodies were incubated for 1 hour at room temperature. The following primary antibodies were used: mouse anti-β-Catenin (610153, BD Transduction Laboratories, 1:100), rabbit anti-active caspase-3 (ab2302, Abcam, 1:100), mouse anti-Ki67 (1:100), rabbit anti-KIM-1 (NBP1-76701, Novus Biologicals, 1:100), rabbit antilaminin (L9393, Sigma, 1:50), goat anti-collagen IV (1340-01, Southern Biotech, 1:50), and rabbit anti-ZO-1 (40-2200, Invitrogen, 1:100). Secondary antibodies purchased from Invitrogen or Life Technologies include: Alexa Flour 488 goat anti-rabbit (1:100 or 1:200), Alexa Flour 633 goat anti-mouse (1:200), donkey anti-mouse 488, donkey anti-goat 488 (1:200), goat anti-mouse 488 (1:200), goat anti-rabbit 633 (1:200). For actin staining, Phalloidin-Atto 633 (68825, Sigma, 1:50) was applied during secondary antibody incubation. Samples were washed and incubated with Hoechst 33342 nuclear dye (Life Technologies) for 20-30 minutes. Resulting samples were then washed 3 times with PBS and imaged.

3.4.3. Confocal microscopy

All fluorescent images were acquired using a Leica confocal SP5 microscope (Leica Mikrosysteme Vertrieb). The following oil objectives were used to acquire images: 20x, 40x, or 63x. Images were captured at 100 or 400 Hz at a resolution of 1024 x 1024 pixels. For the organic anion transporter assay, phase contrast images were also simultaneously acquired using the Leica confocal microscope.

3.4.4. Transmission Electron Microscopy (TEM)

Proximal tubule cell-laden hydrogels were cultured for 4 weeks and then fixed in modified Karnovsky’s fixative (2% glutaraldehyde + 2% paraformaldehyde in 50 mM HEPES) for at least overnight at 4°C. They were washed 2x in 100 mM HEPES and 2x in water, postfixed in 1% OsO4/water for 2hrs on ice, washed several times with water, and en bloc contrasted/postfixed with 1% uranyl acetate/water for 2 hours on ice. The samples were then washed several times in water, dehydrated in a graded series of ethanol/water up to 100% ethanol, infiltrated in epon 812 (epon/ethanol mixtures: 1:3, 1:1, 3:1 for 1.5 hours each, pure epon overnight, pure epon 3 hours), embedded in flat embedding molds and cured at 65°C. Ultrathin sections were prepared with a Leica UC6 ultramicrotome (Leica Microsystems, Vienna, Austria), collected on formvar-coated slot grids,
stained with lead citrate and uranyl acetate as previously described, and analyzed on a FEI Morgagni D268 transmission electron microscope (FEI, Eindhoven, The Netherlands) at 80 kV acceleration voltage. All TEM images and processing after the fixation step were performed by Dr. Thomas Kurth (CRTD, Dresden, Germany).

3.5 Statistical Analysis

Sample size was chosen based on the reproducibility and variability of specific experiments. Samples were only excluded from analysis if they were incubated with assay buffer (ex. LDH reaction mixture) longer than other samples in the same plate due to mechanical error. For all experiments, the same number of cells was embedded in each hydrogel (50,000 cells/hydrogel). For nephrotoxicity studies, experimental (cisplatin treated) and control wells were chosen at random from previously formed hydrogels. Fields and samples for imaging and quantification were chosen at random for all experiments. Investigators were not blinded to the conditions during the experiments or data analysis. All statistics were performed using GraphPad Prism 5 (GraphPad Software Inc.). Multiple samples were analyzed using a one-way analysis of variance (ANOVA) and Tukey’s multiple comparison post-hoc test. To evaluate statistical differences among multiple samples with different parameters, a two-way analysis of variance (ANOVA) and Bonferroni post-hoc test were applied.
Chapter 4

Results and discussion

4.1 Characterization of hydrogel-based tubulogenesis model

The first aim of the thesis was to find the optimal conditions for 3D culture of proximal tubule cells in a hydrogel. To do this, single human immortalized proximal tubule cells (HK-2 cells) were embedded in a range of hydrogel conditions. HK-2 cells were chosen because they have previously been shown to retain many characteristics of primary human proximal tubule epithelial cells, as well as respond to nephrotoxins\textsuperscript{123–125}. Seeding concentrations, mechanical properties, and hydrogel components were systematically varied to find the ideal parameters for the renal tubulogenesis model.

4.1.1 Optimization of cell seeding concentration in 3D culture

The number of cells seeded in culture can make a significant impact on their morphogenesis through paracrine and cell-cell adhesion affects\textsuperscript{31,126}. To determine the optimal seeding concentration to promote the fastest rate of proximal tubulogenesis, two seeding concentrations of HK-2 cells were tested, a “high concentration” of 6 x 10\textsuperscript{6} cells/ml (150,000 cells/gel) and a “low concentration” of 2 x 10\textsuperscript{6} cells/ml (50,000 cells/gel). Due to the known influence of MMPs on renal tubulogenesis (discussed in Section 2.1.3), a MMP cleavable peptide was used as a crosslinker in the starPEG-heparin hydrogels; these hydrogels will be referred to throughout the thesis as starPEG-MMP-heparin hydrogels. The MMP cleavable peptide allows the proximal tubule cells to degrade and remodel the hydrogel matrix through the release of their MMPs.

Tubulogenesis was quantified using nomenclature applied in a previous study, where colonies were classified according to their stage of tubulogenesis; round spheroids were called “smooth spheroids”, spheroids with extensions, a precursor of tubules, were called “spiny spheroids,” and tubules were termed “tubular structures”\textsuperscript{40}. Colonies were considered to be tubular when their length to diameter ratio was greater than 5. In the degradable starPEG-MMP-heparin hydrogels, tubulogenesis was slightly higher in the low cell concentration throughout the study (Fig. 4.1.1. AB). By day 11, there
were significantly less smooth spheroids and more spiny spheroids in the low cell concentration (Fig. 4.1.1. C-E). Therefore, the low cell concentration promotes increased tubulogenesis compared to the high cell concentration and hence, the low cell concentration was used for all following experiments. These results are counter-intuitive, as it would be expected that more cells would lead to a faster rate of tubulogenesis. However, in MMP cleavable hydrogels, proximal tubule cells need to release the adequate amount of MMPs to cleave the hydrogel matrix at a suitable time frame that allows for tubulogenesis, while keeping the hydrogel still intact. A possible explanation for the lower rate of tubulogenesis with the high cell concentration is that the higher number of cells led to more degradation of the hydrogel. Consequently, this would result in a softer hydrogel which was perhaps not conducive to tubulogenesis. The next studies explored this idea further by specifically examining the mechanical effects of the hydrogel on tubulogenesis.
Figure 4.1.1. Effect of seeding concentration on renal tubulogenesis. HK-2 cells were cultured in starPEG-MMP-heparin hydrogels at either a “High cell concentration” (6 x 10^6 cells/ml) or a “Low cell concentration” (2 x 10^6 cells/ml). Tubulogenesis was quantified every two days for the high cell concentration (A) and the low cell concentration (B). Their representative phase contrast images at day 11 are shown below (C, D, respectively). Scale bar, 100 µm. n=3, Mean ± s.d. (E) Tubulogenesis quantification at 11 days to compare between high and low cell concentrations. Mean ± s.d., *P<0.05.
4.1.2 Optimal hydrogel mechanical properties to promote renal tubulogenesis

Numerous studies have revealed the influence of mechanical properties on the survival and proliferation of cells in culture\textsuperscript{127-129}. For example, Chen et al. found that both the chemical composition and physical properties of a matrix influence the differentiation of mouse proximal tubule cells seeded on 2D substrates. Specifically, they found that soft matrices (<1 kPa) promoted the preservation of the epithelial phenotype of proximal tubule cells. They also observed that a normal mouse kidney, measured using Bio-AFM, had an elastic modulus of 300 Pa, although this was an unpublished observation\textsuperscript{130}.

For these reasons, it was important to explore the effect of the hydrogel's mechanical properties on proximal tubule cells and find the optimal stiffness to promote tubulogenesis. To do this, HK-2 cells were embedded in starPEG-MMP-heparin hydrogels with a range of mechanical properties. The stiffness of the hydrogels was tuned by changing the crosslinking degree as previously described\textsuperscript{101,110}. The crosslinking density is expressed as a molar ratio ($\gamma$) of starPEG-MMP to heparin and can be adjusted by changing the ratio of the two components. StarPEG-MMP-heparin hydrogels were formed with a crosslinking degree ($\gamma$) of 0.75 to 1.5. The hydrogels were then measured with a rheometer to find the storage modulus. The rheology data showed that storage modulus (stiffness) of the hydrogels increased with increasing crosslinking density (Fig. 4.1.2.). These results agree with the previous report by Tsurkan et al that also employed starPEG-MMP-heparin hydrogels\textsuperscript{110}.

![Figure 4.1.2. Mechanical properties of starPEG-MMP-heparin hydrogels with different crosslinking densities. The crosslinking degree is expressed as a molar ratio ($\gamma$) of starPEG to heparin. The storage modulus (stiffness) was measured with a rheometer (Mean ± s.d.).](image-url)
The same hydrogel conditions were then applied to cell culture. HK-2 cells were embedded in starPEG-MMP-heparin hydrogels with crosslinking densities of 0.75, 1.0, 1.25, and 1.5 with mechanical properties corresponding to Fig. 4.1.2. Tubulogenesis was quantified in the same manner as described in Section 4.1.1. After 3 weeks in culture, the most tubular structures were observed in the hydrogel with the lowest crosslinking degree ($\gamma = 0.75$) (Fig. 4.1.3A). The stiffest hydrogel ($\gamma = 1.50$) had the least amount of cell colonies that showed signs of tubulogenesis (Fig. 4.1.3.D). The second softest hydrogel ($\gamma = 1.0$) had more than 50% of colonies that underwent tubulogenesis by week 3 (Fig. 4.1.3B). However, this condition did not promote the growth of tubular structures within the duration of the study. Taken together, it was concluded that matrices with a storage modulus $<1$ kPa are optimal for promoting renal tubulogenesis. For future cell studies, hydrogels were made with these mechanical properties.

These findings agree with the previously mentioned paper from Chen et al. which found that softer matrices were ideal for proximal tubule cells and prevented their dedifferentiation$^{131}$. Unfortunately, the results cannot be directly compared to the human kidney because of the limited data on kidney stiffness in the literature. However, one study has used magnetic resonance elastography (MRE) to measure the shear modulus of the kidney. They found a gradient of stiffness within the kidney with the cortex, the region where the majority of proximal tubules reside, to be $\sim 4.35$ kPa$^{132}$.
Figure 4.1.3 Effect of hydrogel mechanical properties on renal tubulogenesis. Tubulogenesis was quantified throughout a 3 week culture. HK-2 cells were embedded in starPEG-MMP-heparin hydrogels at the following crosslinking degrees: 0.75 (A), 1.0 (B), 1.25 (C), 1.50 (D)(Mean ± s.d.). The mechanical properties that correspond to these hydrogels are shown in Fig. 4.1.2.
4.2 Evaluating the effect of hydrogel matrix on PTECs

Once the optimal conditions were established, the influence of individual hydrogel components on renal proximal tubulogenesis was investigated. HK-2 cells were embedded in three hydrogel variants consisting of covalent polymer networks of heparin and/or starPEG, formed as previously described\(^\text{100,101,110}\). To allow the cells to degrade and remodel the hydrogel matrices, a matrix metalloproteinase (MMP) cleavable peptide was attached to the starPEG, as in previous experiments. To explore the influence of heparin and cleavable peptide linkers on renal tubulogenesis, the following conditions were investigated: non-degradable starPEG-heparin hydrogels (without MMP cleavable peptide), degradable starPEG-MMP-starPEG hydrogels (without heparin), and degradable starPEG-MMP-heparin hydrogels.

4.2.1 Influence of hydrogel components on renal tubulogenesis

To create a comparable system, all polymer hydrogels were synthesized with the same mechanical properties (storage modulus of 500 Pa) so that the effect of the composition of the material could be independently investigated (Fig. 4.2.1). Also, all heparin-containing hydrogels had the same amount of heparin (0.56 mg/hydrogel) to have similar biomolecular properties between hydrogels. By keeping the biophysical and biomolecular properties stable between the three hydrogel variants, we were able to determine the unique effect of each hydrogel composition on renal tubulogenesis. Matrigel\(^\text{TM}\), a commercially available reconstituted basement membrane, was used as a control. Matrigel is composed of the extracellular matrix components, laminin, collagen IV, heparan sulfate, entactin, and vitronectin, and is commonly used in studies to coat tissue culture plates or embed cells in 3D matrices\(^\text{49}\).

Tubulogenesis was quantified over a time course of 4 weeks as described in Section 4.1.1. In degradable starPEG-MMP-starPEG hydrogels, proximal tubule cells formed unorganized, round structures (Fig. 4.2.2A), whereas in the non-degradable starPEG-heparin hydrogel, they remained as single cells or formed smooth, round spheroids (Fig. 4.2.2B). Remarkably, in degradable starPEG-MMP-heparin hydrogels, HK-2 cells underwent tubulogenesis and formed complex branched tubule structures by week 4 (Fig. 4.2.2C). Tubulogenesis in starPEG-MMP-heparin hydrogels was significantly higher than the other hydrogel conditions (Fig. 4.2.2D).
Figure 4.2.1. Comparable mechanical properties of tested hydrogel variants. The storage modulus (stiffness) of each hydrogel condition that was used in cell studies was measured by rheometry (Mean ± s.d., n=2-3/condition, measurements performed in triplicate, values not significant (n.s.)).

To further characterize the renal structures in each hydrogel condition, the circularity, surface area, and perimeter of the structures were measured manually using Image J. Circularity is a measurement of roundness where 1.0 corresponds to a perfect circle. The average circularity of renal structures in starPEG-heparin hydrogels was 0.98, whereas structures in starPEG-MMP-heparin hydrogels were significantly less circular, due to their more elongated, tubular structures (Fig. 4.2.2C and Fig. 4.2.3). Renal structures in starPEG-MMP-starPEG hydrogels were less organized which led them to be less circular than structures in starPEG-heparin hydrogels; however, they were still significantly more circular than starPEG-MMP-heparin hydrogels (Fig. 4.2.2A,E). Renal structures in starPEG-MMP-heparin hydrogels were also significantly larger than the other hydrogel conditions (Fig. 4.2.2F, G).
Results and Discussion

Figure 4.2.2. Comparative evaluation of HK-2 cell tubulogenesis and generated renal structures in three hydrogel variants. Quantification of tubulogenesis in the following hydrogel conditions: starPEG-MMP-PEG (A), starPEG-heparin (B), and starPEG-MMP-heparin (C) (Mean ± s.d., n=3/condition). Representative phase contrast images of the corresponding hydrogels are seen below. Scale bar, 100 μm. (D) Compiled tubulogenesis quantification from week 4. (Mean ± s.d., ***P<0.001 compared to starPEG-heparin and starPEG-MMP-starPEG hydrogels). (E) Average circularity of structures in each hydrogel condition, where 1 corresponds to a perfect circle (Mean ± s.d.,
n=3/condition, ***P<0.0005. (F) Surface area of renal structures in each hydrogel condition (Mean ± s.d., n=3/condition, **P<0.01). (G) Perimeter of renal structures in each hydrogel condition (Mean ± s.d, n=3/condition, ***P<0.001).

To verify that heparin was crucial to promote renal tubulogenesis in the hydrogel system, HK-2 cells were embedded in degradable starPEG-MMP-starPEG hydrogels (without heparin) with a range of mechanical properties from 100-500 Pa, correlating to the choice of solid content during gel formation. No signs of tubulogenesis were seen in the starPEG-MMP-starPEG hydrogels (Fig. 4.2.3A). In addition, the softest hydrogel with 2% solid content, was fully degraded by week 4 and showed no signs of tubulogenesis before gel degradation. Therefore, both heparin and a cleavable matrix linker are essentially needed in our hydrogel system to stimulate renal proximal tubulogenesis.

**Figure 4.2.3.** HK-2 cells embedded in starPEG-MMP-starPEG hydrogels with varied mechanical properties. HK-2 cells were embedded in starPEG-MMP-hydrogels with 2, 2.5, and 3% solid content. A) Representative phase contrast images after 4 weeks in culture. StarPEG-MMP-starPEG hydrogels with 2% solid content were fully degraded by week 4. Scale bar, 200 μm. B) Rheology measurements of starPEG-MMP-starPEG hydrogels showing the storage modulus for the solid contents that were used in cell studies.

As a control, HK-2 cells were also embedded in Matrigel, a reconstituted basement membrane from Engelbreth-holm-Swarm mouse sarcoma. Matrigel, has been seen to promote the growth of kidney cells in several studies alone or as a mixture with collagen I. In our studies, HK-2 cells were embedded in Matrigel under similar conditions as starPEG-heparin hydrogels, with 50,000 cells embedded per gel. HK-2 cells formed unorganized structures in Matrigel, similar to renal structures in starPEG-MMP-starPEG hydrogels (Fig 4.2.4). This finding agrees with the study by Santos et al. which showed that MDCK cells cultured in Matrigel with HGF did not form branching tubular
structures like they did in collagen I gels. By dissecting the ECM components in Matrigel and independently testing them on tubulogenesis, they found that laminin, entactin, and fibronectin induced the formation and complexity of tubular structures and collagen IV, heparin sulfate proteoglycan, and vitronectin, inhibited tubular formation. Therefore, it is possible that the amount of these inhibitory ECM components, are presented too high in Matrigel to allow for proximal tubule formation.

![Matrigel](image)

**Figure 4.2.4. Representative image of HK-2 cells cultured in Matrigel.** HK-2 cells were embedded in Matrigel in the same conditions as for synthetic hydrogels. Phase contrast image taken after 4 weeks in culture. Scale bar, 100 μm.

To test the reproducibility of this model, the study was repeated with a second cell line, conditionally immortalized human proximal tubule epithelial cells (ciPTEC). CiPTEC were isolated from urinary sediment of a healthy donor and immortalized with SV40T and hTERT, as previously described. This allows them to be maintained for at least 45 passages when grown at 33°C. When cultured at 37°C for 10 days, CiPTEC express multiple drug transporters and maintain morphological characteristics of the native human proximal tubule. CiPTEC cells were embedded in the three synthetic hydrogel variants under the same conditions as HK-2 cells, with 50,000 cells/hydrogel.

Results from ciPTEC studies showed a similar trend of forming round spheroids in non-degradable starPEG-heparin hydrogels and significantly more tubule structures in the degradable starPEG-MMP-heparin hydrogels (Fig. 4.2.5A,B), confirming the results from HK-2 cells. CiPTEC cells formed smaller tubule-like structures than HK-2 cells after 4 weeks in culture; this could be due to their difference in origin. CiPTEC cells were isolated from urinary sediment whereas HK-2 and primary proximal tubule cells were obtained from human tissue. Another difference between ciPTEC and HK-2 cells was seen in degradable starPEG-MMP-starPEG hydrogels, where ciPTEC spontaneously underwent apoptosis after a few days in culture. This could be caused from the pH of
starPEG-maleimide that needs to be adjusted to 4-5 to decrease the time of gelation when forming starPEG-MMP-starPEG hydrogels. CiPTEC cells resemble human primary proximal tubule cells at 37°C and hence, are inherently more sensitive than traditional cell lines. Therefore, it is possible that the low pH in the starPEG-maleimide solution directed them to undergo apoptosis. CiPTEC had a significantly higher metabolic activity in degradable starPEG-MMP-heparin hydrogels compared to other hydrogel conditions (Fig. 4.2.5C). Taken together, CiPTEC results confirm that the hydrogel-based model is reproducible with other proximal tubule epithelial cells.

Figure 4.2.5. Comparative evaluation of CiPTEC cells embedded in three different hydrogel variants. Tubulogenesis quantification of CiPTEC cells in non-degradable starPEG-heparin (A) and non-degradable starPEG-MMP-heparin (B) (Mean ± s.d., n=3-4/condition, >100 colonies/condition, experiments performed in duplicate). Representative phase contrast images of CiPTEC cells after 4 weeks in culture seen below. Tubulogenesis was not quantified in starPEG-MMP-starPEG hydrogels due to cell death. Scale bar, 200 μm. (C) Metabolic activity of CiPTEC cells after 4 weeks in culture (Mean of pooled experiments ± s.e.m., n=3-4/condition, experiments performed in duplicate; ***P<0.0001).
4.2.2 Effect of hydrogel components on cell viability

To further understand how the hydrogel components affect proximal tubule cells, metabolic activity and lactate dehydrogenase (LDH) release were measured after HK-2 cells were cultured in the hydrogels for 4 weeks. Metabolic activity is the sum of the chemical reactions that occur in a cell. LDH is an enzyme released by cells following damage to the plasma membrane, and can be used to quantify necrotic cells\textsuperscript{133}. Hence, it is often used in cytotoxicity assays.

Metabolic activity of HK-2 cells was significantly higher in degradable starPEG-MMP-heparin hydrogels compared to all other hydrogel conditions (Fig. 4.2.6A), similar to ciPTEC cells (Fig. 4.2.5C). This data suggests that cell activity is increased from hydrogels incorporated with heparin and MMP-cleavable peptides, which could contribute to their resulting tubulogenesis. HK-2 cells in Matrigel had a significantly higher metabolic activity than starPEG-MMP-starPEG and starPEG-heparin hydrogels (Fig. 4.2.6A). Matrigel consists of natural ECM components from an animal source and it is therefore, not surprising that it induces a higher metabolic activity compared to synthetic hydrogels. Interestingly, with the addition of heparin and a cleavable matrix, it was possible to engineer a hydrogel that promotes higher metabolic activity than a natural ECM derived hydrogel (Fig. 4.2.6A).

HK-2 cells cultured in degradable starPEG-MMP-heparin hydrogels also had significantly less LDH release than starPEG-MMP-starPEG and Matrigel conditions. Although starPEG-heparin hydrogels had a low metabolic activity, they also had a significantly low LDH release. It is possible that because the HK-2 cells in starPEG-heparin hydrogels are unable to degrade the matrix, it triggers them to become quiescent which results in a lower metabolic activity. It is interesting that the two hydrogels containing heparin have the lowest LDH release. However, it is important to note that starPEG-MMP-starPEG hydrogels and Matrigel had the most cells that evaded the hydrogels and grew on the bottom of the tissue culture plate, potentially contributing to their high LDH levels. They also were the only samples where media changed to yellow in between media changes, signifying a change in pH.
Figure 4.2.6. Metabolic activity and LDH release of HK-2 cells in response to synthetic and naturally derived hydrogels. HK-2 cells were embedded in hydrogels and cultured for 4 weeks. A) Metabolic activity measured with PrestoBlue assay (Mean of pooled experiments ± s.d., n =3/condition, experiments performed in triplicate, ***P<0.0001, starPEG-MMP-heparin significant vs. all other conditions). B) Cytotoxicity measured by lactate dehydrogenase (LDH) levels in supernatant after 4 weeks in culture (Mean of pooled experiments ± s.d., n=2-3/condition, experiments performed in duplicate, ***P<0.0001).

To more closely understand the viability of the renal structures, the amount of proliferating and apoptotic cells in the structures was analyzed. Renal structures from each hydrogel condition were stained with Ki67, a proliferation marker, and cleaved caspase-3, a marker of apoptosis. Tubule structures in degradable starPEG-MMP-heparin hydrogels contain multiple proliferative cells, and limited apoptotic cells (Figure 4.2.7). Spheroids in starPEG-heparin hydrogels showed more apoptotic cells than other conditions that localized to the lumen (Fig. 4.2.7). The formation of lumens by apoptosis is a common mechanism that has previously been observed in the generation of cysts and tubules\(^46\). Therefore, the increase of apoptotic cells in spheroids is most likely due to this process. Surprisingly, although starPEG-MMP-starPEG hydrogels promote the formation of unorganized colonies, their structures contain both proliferating and apoptotic cells, signifying that the matrices support the growth of proximal tubule cells, although they do not contain any biomolecular properties to stimulate tubulogenesis.
Figure 4.2.7. IF of proliferation and apoptotic markers in renal structures after 4 weeks in culture. Confocal images showing the expression of the proliferation marker, Ki67, and the apoptotic marker, cleaved caspase-3 in representative structures in each hydrogel condition. Insets are magnified views of structure. Scale bar, 75 μm. Inset scale bar, 25 μm.
4.2.3 Evaluation of polarization and architecture of renal structures

The *in vivo* human proximal tubule is characterized by a polarized, cuboidal epithelium, with many mitochondria on the basal side, Golgi apparatus localized to the apical side, a primary cilium, and a brush border made up of microvilli that faces the lumen of the tubule\textsuperscript{21,134}. These characteristics are portrayed in Fig. 4.2.8.

![Figure 4.2.8. Schematic of polarized proximal tubule and a representative cell. Cilium, Golgi, M, mv, and N represent primary cilium, Golgi apparatus, mitochondria, microvilli, and the nucleus, respectively.](image)

To evaluate polarization and morphology, renal structures in all three synthetic hydrogel conditions were analyzed by transmission electron microscopy (TEM) and IF. Renal structures were evaluated for the previously mentioned signs of polarization. TEM can capture images at significantly higher resolution than light microscopes. Using this technique, we were able to examine the ultrastructure of the cells. HK-2 cells were embedded in the three hydrogel conditions and then cultured for 4 weeks. They were then fixed and processed for TEM imaging which can be seen more in detail in Materials and Methods section.

In degradable starPEG-MMP-starPEG hydrogels, HK-2 cells showed no signs of polarization with cells forming unorganized structures with no lumens (Fig. 4.2.9A). In contrast, in starPEG-heparin hydrogels, HK-2 cells formed spheroids with lumens, often filled with cell debris (Fig. 4.2.9B). These cells had a cuboidal-like epithelial phenotype, however, not all Golgi apparatuses were localized to the apical side (Fig. 4.2.9B) or are fully aligned (Fig. 4.2.10A), but they did contain some microvilli (Fig. 4.2.10B). Interestingly, tubular structures formed in degradable starPEG-MMP-heparin hydrogels had a cuboidal epithelium with clear lumens, Golgi apparatus localized to the apical side, and basal mitochondria (Fig. 4.2.9C and Fig. 4.2.10E). The tubules did not possess a brush border
but did contain some microvilli, as well as primary cilium (Fig. 4.2.10C-E). Remarkably, these generated tubular structures recapitulated the diameter of human proximal tubules which published reports observed to have a diameter of 30-60 μm\(^{23,135}\). In addition, the height of the HK-2 cells in the tubular structure in Fig. 4.2.9C was measured (using Image J) to be \(~9\) μm, whereas the average cell height in physiological human proximal tubules is \(~8\) μm\(^{135}\). Therefore, the tubular structures generated in starPEG-MMP-heparin hydrogels recapitulate the physiological size of the human proximal tubule.

**Figure 4.2.9.** Electron micrographs of generated renal structures in three different hydrogel variants. HK-2 cells were cultured in 4 weeks in (A) degradable starPEG-MMP-starPEG, (B) non-degradable starPEG-heparin, and (C) degradable starPEG-MMP-heparin hydrogels with progressive magnifications of selected regions below. Scale bar of enlarged region, 10 μm. Scale bar of magnified regions, 1 μm. Cilium, Golgi, M, mv, and N represent primary cilium, Golgi apparatus, mitochondria, microvilli, and the nucleus, respectively.

A few structures in starPEG-MMP-heparin hydrogels did not have the thick cuboidal cells like shown in Figure 4.2.9 but were thinner and stretched around a lumen. Remarkably, these structures have a strong resemblance to the squamous epithelia of the thin limb of the loop of Henle (Appendix Fig. 7.1). It is possible that the source of HK-2 cells contain a small pool of cells from the
loop of Henle since HK-2 cells are originally isolated from heterogeneous tissue of the kidney cortex\textsuperscript{123,136}. It is fascinating that within a heterogeneous population of cells, cells with the same phenotype migrate to each other in the hydrogels and form structures mimicking their \textit{in vivo} architecture. These results strongly suggest that proximal tubule cells and other tubular epithelial cells of the nephron have an inherent ability to undergo tubulogenesis which can be stimulated with starPEG-MMP-heparin hydrogels. In the future, it would be interesting to embed a heterogeneous mixture of renal tubule epithelial cells in the starPEG-MMP-heparin hydrogels to evaluate the generated structures and see if structures from the different cell types attach together to form a physiologically-relevant nephron tubule.

![Figure 4.2.10 Electron micrographs of the ultrastructure of HK-2 cells cultured in hydrogels.](image)

HK-2 cells were cultured in non-degradable starPEG-heparin hydrogels (A, B) and degradable starPEG-MMP-heparin hydrogels (C-F). Electron micrographs show the ultrastructure of cells after 4 weeks in culture. Cilium, Golgi, M, mv, and N represent primary cilium, Golgi apparatus, mitochondria, microvilli, and the nucleus, respectively. Scale bars, 1 μm

To determine if HK-2 cell derived renal structures form cell-cell junctions, a further characteristic of polarization, structures were stained for β-catenin. β-catenin is a protein associated with the cytoplasmic region of E-cadherin and plays a role in cell-cell adhesion. It is located basolaterally on polarized proximal tubule cells. Renal structures in each hydrogel condition were stained for β-
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catenin after a culture time of 4 weeks. β-catenin could be seen on the basolateral sides of HK-2 cells in tubule structures in starPEG-MMP-heparin hydrogels and in spheroids in starPEG-heparin hydrogels (Fig. 4.2.11). The protein could also be seen in cells in colonies formed in starPEG-MMP-starPEG hydrogels, although the staining might be slightly less uniform. These results support the study by Chung et al. that found that PEG hydrogels with a similar MMP cleavable peptide promote the formation of unorganized colonies of MDCK cells. Specifically, the MDCK cell-derived colonies had an inverted polarization and less defined lumens compared to colonies in PEG hydrogels functionalized with laminin or RGD<sup>137</sup>. These results support the data from this thesis that show that additional cell attachment sequences are needed to promote the formation of polarized structures.

To further evaluate the architecture of the structures, it was necessary to determine if renal basement membranes similar to native proximal tubules were present. The renal basement membrane is an important part of the proximal tubule and has been shown to have a role in differentiation, cell adhesion, and filtration<sup>50</sup>. To explore if HK-2 cells created their own extracellular matrix <i>in vitro</i>, the renal structures were stained for collagen IV and laminin, the main components of the proximal basement membrane<sup>50</sup>. Collagen IV and laminin could be seen around the perimeter of renal structures in starPEG-heparin and starPEG-MMP-heparin hydrogels (Fig. 4.2.12). Surprisingly, unorganized structures in starPEG-MMP-starPEG hydrogels also had collagen IV around structures, but had noticeably less laminin than other conditions (Fig. 4.2.12).

This data suggests that all hydrogels promote an environment where HK-2 cells can create their own ECM. The ECM was localized on the outside of the structures, similar to the renal basement membrane <i>in vivo</i>, which coats the outer surface of the tubule. The ECM was mostly uniformly distributed around spheroids in starPEG-heparin conditions. This could be due to their more simple structure, which also makes them easier to image compared to tubule structures in starPEG-MMP-heparin hydrogels. Although these findings suggest that HK-2 cells formed a basement membrane <i>in vitro</i>, this has not been confirmed by TEM images and so a conclusion cannot be made. More magnified TEM images should be taken to fully elucidate the presence of a full basement membrane. However, from this data we can say that the cells create their own ECM in the hydrogels and are more polarized in heparin-containing hydrogels. The only condition that generated polarized tubule structures that resembled the polarization and architecture of the <i>in vivo</i> proximal tubule was the degradable starPEG-MMP-heparin hydrogels. For these reasons, it was determined that starPEG-
MMP-hydrogels are the optimal matrix for a tubulogenesis model and future studies were conducted using this condition.

Figure 4.2.1. IF of polarization markers of renal structures after 4 weeks in culture. Confocal images showing the basolateral marker, β-catenin, in representative structures in each hydrogel condition. Insets are magnified views of structure. Scale bar, 75 μm. Inset scale bar, 25 μm.
Figure 4.2.12. IF of extracellular matrix deposition after 4 weeks in culture. Confocal images showing representative HK-2 cell-derived structures in each hydrogel condition. Insets are magnified views of structure. Scale bar, 75 μm. Inset scale bar, 25 μm.
4.3 Effect of soluble cues on renal tubulogenesis

Tubulogenesis is a complex process that involves the coordination of various soluble factors such as cytokines, growth factors, proteases, and extracellular matrix components. From the previous studies it has been determined that the optimal hydrogel composition to promote renal tubulogenesis is the degradable starPEG-MMP-heparin hydrogels. Having fully established and characterized the hydrogel model, it was then used to explore tubulogenesis to show the versatile capabilities of the model and demonstrate that it can potentially be used for developmental biology, disease modeling, and regenerative medicine applications.

4.3.1 Influence of growth factors in FBS on tubulogenesis

Fetal bovine serum (FBS) is the blood fraction that remains after the coagulation of whole blood and the separation from red blood cells via centrifugation. It contains a complex mixture of proteins, growth factors, hormones, nutrients, trace elements, and is a common supplement to cell culture media. The various factors are often essential for the growth and maintenance of cells in vitro. The purpose of this study was to determine if fetal bovine serum was required to promote renal tubulogenesis using the established hydrogel model and if so, are the growth factors in serum the stimulating components.

The first study was designed to conclude if fetal bovine serum had an effect on renal tubulogenesis in the starPEG-MMP-heparin hydrogels. Three concentrations of FBS were applied to the cultures: 0, 5, and 10%. HK-2 cells were embedded in the hydrogels and the samples were cultured for 3 weeks. Tubulogenesis was quantified weekly and metabolic activity was measured at 3 weeks. In the 0% FBS condition, tubulogenesis did not occur, with the majority of cells (91%) remaining rounded by week 3 (Fig. 4.3.1A). In both FBS treated conditions, HK-2 cells underwent tubulogenesis (Fig. 4.3.1A). By week 3, samples with 10% FBS had an overall higher tubulogenesis than 5% FBS when spiny spheroids, precursors of tubules, were taken into account (Fig. 4.3.1B). Samples with 5% and 10% FBS had significantly less smooth spheroids and significantly more spiny spheroids compared to the 0% FBS control (Fig. 4.3.1B). Therefore, FBS has a direct effect on renal tubulogenesis.

As expected, FBS also had a significant impact on the metabolic activity of HK-2 cells embedded in the degradable hydrogel model. A concentration dependent response was shown with both 5% and
10% FBS having a significantly higher metabolic activity compared to the 0% control, and 10% FBS having the highest effect (Fig. 4.3.1C). This agrees with many studies where FBS was reported to support growth, increase size, and/or stimulate proliferation of a range of cell types\textsuperscript{138-141}. The FBS-induced increase in metabolic activity could contribute to the higher rate of tubulogenesis seen in the FBS-treated hydrogels by stimulating the cells to be more active, thereby forming tubule structures.

Other studies have also shown that the presence of FBS or specific growth factors stimulates renal tubulogenesis in 3D cultures\textsuperscript{57,95,142}. Although FBS contains many proteins such as adhesion proteins, extracellular components, and hormones that have all been shown to effect cell behavior\textsuperscript{143,144}, it is hypothesized that growth factors in the serum are key modulators of tubulogenesis in culture.

**Figure 4.3.1.** Influence of fetal bovine serum on renal tubulogenesis and metabolic activity. Tubulogenesis was quantified over a time course of 3 weeks (A) (Mean ± s.d., n=3). B) Compiled tubulogenesis at week 3 (Mean ± s.d., n=3, ***P<0.001, *P<0.05). C) Metabolic activity at week 3 (Mean ± s.d., n=3, **P<0.005, *P<0.05).

To test this hypothesis and further understand the specific components in FBS that contribute to renal tubulogenesis, a serum substitution experiment was performed. To do this, panexin NTA, a serum substitute from PAN Biotech GmbH, was added to the culture. Panexin NTA is a chemically defined composition of purified proteins, lipids, salts, amino acids, trace elements, attachment factors, and hormones that simulate serum composition. The substitute does not contain any growth
factors, therefore, it can be used to determine if the presence of growth factors in the serum play a role in stimulating renal tubulogenesis.

Panexin NTA was added to DMEM/F-12 medium with 1% P/S at 10% v/v. 10% FBS was used as a control. The medium for both conditions came from the same bottle to ensure that all other experimental factors were the same. In addition, all of the cells used in the study were from the same passage. The samples were monitored over time and tubulogenesis was quantified. After 1 week in culture, the majority of HK-2 cells with the serum substitute remained round with only about 25% progressing to spiny spheroids (Fig. 4.3.2A). However, by week 2 cells with NTA looked unhealthy with signs of cell death, resulting in the amount of round cells increasing. For this reason, tubulogenesis was not quantified at week 3 for the NTA condition. On the contrary, HK-2 cells with 10% FBS in the medium displayed the usual evolution of tubulogenesis, comparable to results from the previous FBS study (Fig. 4.3.2A).

**Figure 4.3.2. Effect of serum substitute on renal tubulogenesis.** NTA is a commercially available serum substitute that contains no growth factors. A) Tubulogenesis quantification. (Mean ± s.d., n=3 condition, >100 colonies/condition) (B) Metabolic activity of HK-2 cells after 4 weeks in culture (Mean ± s.d., n = 3, *P<0.05.).

Metabolic activity was also measured at week 3 to determine the effect of serum growth factors on cell activity. Proximal tubule cells in FBS treated samples were significantly more metabolically active (~10 times more) than NTA samples (Fig. 4.3.2B). It should also be noted that hydrogels in the NTA condition were much softer at the end of the study and 2 out of 3 hydrogels were degraded and contained dead cells. The probable reason for this is that cells without growth factors underwent programmed cell death, potentially necrosis. Necrosis is caused by physico-chemical stress and is characterized by cytoplasmic swelling, plasma membrane damage, and organelle breakdown. Upon
membrane damage, the cellular contents can seep out into the extracellular environment. Therefore, if HK-2 cells underwent necrosis they would have released their extracellular contents, which would include MMPs. Proximal tubule cells have been shown to produce MMP-2, 3, 7, 9, 14, and 24. As previously mentioned, starPEG-MMP-heparin hydrogels contain a degradable peptide with the sequence GPQG↑↓IWGQ which is cleavable by MMP-1, 2, 3, 7, 8, and 9. Thus, it is probable that when HK-2 cells become necrotic they release MMPs that degrade the hydrogel matrix, contributing to the hydrogels being softer, compared to hydrogels that were cultured with FBS.

From these serum studies, it was determined that the growth factors in FBS are necessary to maintain HK-2 cell viability and promote tubulogenesis in the hydrogel-based renal model. These results agree with findings from Sakurai et al. that show that multiple growth factors modulate tubulogenesis in vitro. The growth factors HGF, EGF, insulin growth factor (IGF-1), and fibroblast growth factor (FGF-2) have all been seen to have a positive impact on tubulogenesis and have all been found in serum. Sakurai et al. found that a growth factor mix and mouse embryonic kidney cell conditioned media were the only conditions to promote UB cells to form tubules, whereas single growth factors alone did not have the same effect.

### 4.3.2 Influence of HGF on tubulogenesis and its binding to hydrogels

The previous results showed that growth factors are needed in the hydrogel model to instigate renal tubulogenesis. Now that 10% FBS was found to be the optimal condition to promote tubulogenesis, it was important to next determine if tubulogenesis could be further modulated through the addition of growth factors to the culture.

HGF, also known as scatter factor, is a protein found in serum that has been demonstrated to stimulate the growth of proximal tubule cells in vitro. It is a high molecular weight (70-100 kDa) heparin-binding protein that stimulates epithelial proliferation, motility, morphogenesis, and angiogenesis in various organs. It has also been seen to have a major role in renal tubulogenesis. Several studies have found that tubule cells embedded in collagen I matrices required HGF to undergo tubulogenesis. When HGF was not added to the cultures, the tubule cells remained rounded. Therefore, the established hydrogel-based renal tubulogenesis model presented in this thesis is already an improvement on prior naturally based ECM models made with collagen and...
Matrigel. The goal of this experiment was to determine if the addition of HGF to our system would increase the growth of proximal tubule cells and lead to a faster rate of tubulogenesis.

Bowes et al. found that the concentration of HGF that enhanced maximal proliferation of primary renal proximal tubular rat cells in a collagen matrix was between 5-20 ng/ml. Consequently, three HGF concentrations were chosen on the same range, 2, 20, and 200 ng/ml. HGF was added both to the hydrogels upon fabrication and in the media throughout the culture period to limit the diffusion of HGF out of the hydrogels. HGF was embedded in the hydrogels by premixing with the heparin-maleimide solution. HGF contains heparin binding sites and so upon mixing, it binds to the heparin and can then be released from the hydrogel over time. The HGF-heparin solution was then mixed with the HK-2 cells and subsequently mixed with starPEG-MMP to form the hydrogels, following the standard protocol. HGF was added to DMEM/F-12 medium with 1% P/S and 10% FBS. The medium was changed every 3 days and the study was in culture for 2 weeks.

**Figure 4.3.3. Effect of HGF on renal tubulogenesis.** HGF was embedded in the hydrogels at 4 different concentrations: 0, 2, 20, and 200 ng/ml. A) Tubulogenesis quantification of HK-2 cells at week 2 (Mean ± s.d., n=2-3/condition, >100 colonies/condition, **P<0.01, *P<0.05). B) Metabolic activity of HK-2 cells at week 2 (Mean ± s.d, n=3/condition, **P<0.01, *P<0.05).

Tubulogenesis was quantified and is displayed in Fig. 4.3.3A. The only HGF concentration that stimulated a significant increase in tubulogenesis was the highest concentration, 200 ng/ml. Samples with 200 ng/ml had significantly more spiny spheroids than the control (0 ng/ml HGF) and
significantly less smooth spheroids compared to all conditions (Fig. 4.3.3A). There was no substantial difference between the control (0 ng/ml HGF) and 2, and 20 ng/ml HGF.

Metabolic activity of HK-2 cells in the HGF treated cultures was also measured at week 2. Results show that HGF triggered a concentration dependent increase in metabolic activity (Fig. 4.3.3B). The highest concentration of HGF applied (200 ng/ml) resulted in HK-2 cells that had a significantly higher metabolic activity compared to the control and 2 ng/ml HGF. These results agree with findings from Bowes et al. where HGF induced a concentration dependent increase in metabolic activity, measured by MTT assay, when added to the culture of primary rat proximal tubule cells embedded in collagen. They also found that when applied separately to media with 10% FBS, HGF led to significantly more cells than the FGF-1, EGF, IGF-1, FGF-7, and TGF-β1 when added alone. Interestingly, when HGF was combined with other growth factors, there was an induced proliferation compared to the concentration of HGF alone. Therefore, it is possible that combining HGF with another growth factor would induce an increase in proliferation and tubulogenesis. These studies were performed using 20 ng/ml HGF. However, in the starPEG-MMP-heparin hydrogel tubulogenesis model, 20 ng/ml HGF did not illustrate a change in tubulogenesis. One potential reason for this is that HGF contains high affinity binding sites to heparan sulfate and heparin, whereas collagen I has low affinity binding sites for HGF.

The structure and amount of HGF binding in the system can contribute to the bioactivity of the growth factor. For example, Sakata et al. found that in the presence of heparin, HGF has an increased tendency to oligomerize. If this oligomerization is important for signal transduction, then the addition of heparin to the system could affect the bioactivity of HGF. Indeed, Bowes et al. found that the addition of 10 μg/ml heparin to 3D collagen cultures initiated the maximal HGF-induced proximal cell proliferation. Although the concentrations of 2 and 20 ng/ml did not affect tubulogenesis in the established model, it did affect the metabolic activity of the cells. Therefore, it is possible that although the rate of tubulogenesis was only changed with the highest concentration, HGF might have altered the amount of branches or size of the structures. Unfortunately, this was not quantified but future studies could be done to see how HGF affects tubule branching and size in the hydrogels.
To better understand how HGF from serum interacts with the hydrogels, a growth factor binding study was performed with the hydrogels. Unfortunately, the amount of HGF in FBS cannot currently be measured due to a lack of bovine antibodies and so, umbilical cord blood serum was used for the study. It is assumed that this concentration would be similar in FBS, as several studies have revealed that umbilical cord serum can be used to replace FBS in *in vitro* cultures of foreskin fibroblasts, mesenchymal stem cells, and endothelial cells\(^{155-157}\). The measured concentration of HGF in human umbilical cord blood was found by Patki et al. to be \(2.5816 \pm 0.10828\) ng/ml\(^{151}\). To confirm these results, this experiment was repeated in our lab using a Multiplex assay, where it was found that serum contained \(~2\) ng/ml HGF (Fig. 4.3.4). It should be noted that this was repeated with a second batch of cord serum that contained a HGF concentration of 1.3 ng/ml (data not shown), which confirms literature that states serum has batch-to-batch variations, which can alter results\(^{158}\). Nevertheless, it is useful to understand the approximate amount of HGF in serum and how it interacts with starPEG-heparin hydrogels.

To simulate the HK-2 cell experiments, 10% serum in DMEM medium was also measured. The samples were measured fresh at time 0 and after 24 hours incubation at room temperature. For 100% serum samples, the HGF level increased after 24 hours, this occurred for both batches of serum tested. A possible reason for this is that the many proteins in serum aggregated over the 24 hours at room temperature, which caused a higher fluorescent level, and thus a higher read out. However, the 10% serum samples decreased after 24 hours or stayed the same when incubated with heparin. This decrease could be from growth factor degradation during the incubation time. In contrast, heparin has been seen to protect and stabilize growth factors\(^ {112}\). Thus, when incubated with 10% serum, heparin might protect HGF from degrading and be the reason that the HGF concentration remained stable after the 24 hour incubation (Fig. 4.3.4).

Next, the amount of HGF that binds to hydrogels in culture was examined. To do this, 10% cord serum in DMEM medium was incubated with the hydrogels for 24 hours at room temperature and then the supernatant was removed and the unbound HGF was measured. A low \((\gamma = 0.63)\) and high \((\gamma = 1.0)\) crosslinked starPEG-heparin hydrogel was used for the study to determine how pore size affects HGF binding. Collagen gels were used as a control matrix because they offer a simplistic model that exhibits weak binding to proteins\(^ {159,160}\). Two different collagen concentrations (0.9 and 3 mg/ml) were also used. Interestingly, there was negligible difference between the low and high
crosslinking density of both types of hydrogels, signifying that in the conditions tested, pore size did not affect HGF binding. The results show that as expected, the collagen gels did not bind HGF. In contrast, the supernatant from starPEG-heparin hydrogels contained less HGF than 10% cord serum at time 0, suggesting that the difference is from HGF binding to the hydrogels (Fig. 4.3.4). Based on these results, about 1/3 of HGF in 10% serum binds to the hydrogels.

Previously reported studies found that HGF in the presence of heparin did not alter its binding to the cell receptor c-MET, indicating that bound HGF remains bioactive and even may enhance mitogenic potency of the growth factor\textsuperscript{161}. Therefore, it is possible that the amount of HGF in 10% FBS alone, could be enough to stimulate tubulogenesis in the starPEG-MMP-heparin hydrogels. If the system is already saturated with HGF, PTECs would not be sensitive to additional HGF added to the system.

![Figure 4.3.4](image-url)

**Figure 4.3.4. HGF levels in human umbilical cord serum and its binding to hydrogels.** HGF concentration was measured in 100% serum or 10% serum in DMEM medium. The binding of HGF to hydrogels was also measured by incubating 10% serum with two different crosslinked starPEG-heparin hydrogels and two different collagen gel concentrations (Mean ± s.d.). StarPEG-heparin hydrogel 1 and 2 correspond to the crosslinking degree 0.63, and 1.0, respectively. Collagen 1 and 2 correspond to collagen gels made with 0.9 mg/ml and 3 mg/ml. Solid bars indicate HGF level after 24 hours of incubation at room temperature (t = 24) and dashed bars indicate the level at time 0 (t = 0).
To test this theory, another HK-2 cell experiment was conducted in low serum conditions (5% FBS). Two HGF concentrations were examined, 20 and 200 ng/ml. HGF was embedded in hydrogels and supplemented to the media in the same way as the previous experiment. As controls, DMEM/F-12 media with 5% FBS and 10% FBS, the established standard condition, with no HGF was used. Samples were kept in culture for 4 weeks. Interestingly, in the lower FBS condition, 20 ng/ml HGF was able to increase the tubulogenesis that was seen at week 4 (Fig. 4.3.5A) with significantly less smooth spheroids and significantly more tubular structures compared to both 0 ng/ml HGF with 5% FBS and the standard, 0 ng/ml HGF with 10% FBS. This indicates the importance of HGF in promoting tubulogenesis and reveals that it may be more critical than serum. The highest concentration of HGF also had significantly less smooth spheroids compared to the controls, signifying that more colonies were undergoing tubulogenesis in this condition.

Metabolic activity was also examined at week 4. Although 20 ng/ml HGF had a substantial effect on renal tubulogenesis, it only slightly increased metabolic activity of HK-2 cells compared to the 5% FBS without HGF, and interestingly had a similar metabolic activity to 10% FBS without HGF (Fig. 4.3.5B). The highest concentration of HGF (200 ng/ml) promoted a significantly higher metabolic activity compared to all other conditions (Fig. 4.3.5B). This agrees with our previous results that showed that HGF stimulates the metabolic activity of HK-2 cells (Fig. 4.3.3.B) and demonstrates that 10% FBS can be replaced in our hydrogel system with 5% FBS supplemented with 20 ng/ml HGF to have a similar metabolic activity and an increased rate of tubulogenesis. However, this study should be repeated to confirm these results.
4.3.2 Impact of MMPs on renal tubulogenesis

Now that it has been established that tubulogenesis and metabolic activity of HK-2 cells in starPEG-MMP-heparin hydrogels can be enhanced with growth factors, the next aim was to explore how tubulogenesis can be inhibited. Renal tubulogenesis occurs through cell proliferation, migration, differentiation, and degradation of ECM\textsuperscript{46}. In addition to growth factors, there are many other proteins present in the extracellular renal environment, secreted by proximal tubule cells and other cells in proximity. One type of protein commonly found in the epithelial extracellular environment are MMPs. MMPs are endopeptidases that are involved in remodeling of the extracellular matrix and secreted by a variety of cell types including proximal tubule cells and endothelial cells. As stated earlier, proximal tubule epithelial cells secrete MMP-2, 3, 7, 9, 14, and 24\textsuperscript{146}. Several studies have revealed that released MMPs play a direct role in tubulogenesis\textsuperscript{162,163}. The aim of this study was to investigate if tubulogenesis could be repressed by inhibiting MMP release from proximal tubule cells.

A synthetic small molecule broadband MMP inhibitor (GM6001, Millipore) was used for the study. (MW = 388.47). The inhibitor was added to the medium and incubated with the hydrogel samples at
a concentration of 20 μM. As a control, PBS was added to the medium in untreated samples. The samples were cultured for 3 weeks to determine the effect on renal tubulogenesis.

![Graph showing influence of MMPs on HK-2 tubulogenesis and metabolic activity.](image)

**Figure 4.3.6. Influence of MMPs on HK-2 tubulogenesis and metabolic activity.** A commercially available broadband MMP inhibitor was added to medium for the culture of the tubulogenesis model. A) Tubulogenesis quantification. (Mean ± s.d., n=3 condition, >100 colonies/condition) (B) Metabolic activity of HK-2 cells after 3 weeks in culture (Mean ± s.d., n = 3).

The quantification of renal tubulogenesis shows that the addition of the MMP inhibitor significantly impedes tubulogenesis of HK-2 cells cultured in starPEG-MMP-heparin hydrogels. By week 1, there was a considerable difference between the percent of spiny spheroids in the standard conditions (control) and the inhibitor treated samples (60% vs. 20% spiny spheroids) (Fig. 4.3.6A). While spiny spheroids in the controls started to form tubule structures by week 2, the amount of spiny spheroids in the treated samples remained constant compared to week 1. Interestingly, although the tubulogenesis was dramatically different between the two conditions, the metabolic activity of HK-2 cells cultured in both conditions was not significantly different (Fig. 4.3.6B). However, the standard deviation was very high between the MMP inhibitor treated samples meaning that the metabolic activity results might not be entirely reliable. Indeed, 2 out of 3 gels cultured with the MMP inhibitor had unhealthy cells by week 3, the remaining hydrogel had healthy rounded cells. The MMP inhibitor effectively inhibited tubulogenesis in the hydrogel model. Therefore, it can be concluded that HK-2 cells release MMPs and these MMPs are crucial for the formation of tubule structures in starPEG-MMP-heparin hydrogels.

This conclusion is confirmed by a previously reported human renal tubulogenesis study that found that the addition of the endogenous tissue inhibitors of metalloproteinases, TIMP-1 and TIMP-2, to
 proximal tubule cells in a collagen/Matrigel matrix significantly reduced the number of tubular structures. However, in their study, endothelial cells were co-cultured with the proximal tubule cells or cultured with their conditioned media, which they showed to contain MMP-1, 2, and 3. They did not test the proximal tubule cell media for the presence of MMPs and the cells also required HGF to form tubules. In contrast, our established hydrogel-based model suggests that proximal tubule cells may produce their own MMPs which affect the ability of the cells to undergo tubulogenesis, probably through a combination of autocrine and paracrine effects. Nevertheless, it should be noted that FBS contains many proteins and proteases which could be another source of the MMPs found in the hydrogel culture. To prove that the MMPs in our culture system are produced from the cells, further studies should be done with FBS pretreated with a MMP inhibitor.

Taken together, it can be assumed that MMPs in culture help to cleave the hydrogel matrix through the MMP sensitive peptide sequence. This then allows the proximal tubule cells to remodel the surrounding matrix and give them space to form tubule structures. This theory is supported by the work from previously reported studies which demonstrated that MMP-1 and MMP-2 gave MDCK cells the ability to penetrate collagen matrices and accelerate or modify tubulogenesis. Our study confirmed that MMPs play an important role in orchestrating the complex process of renal tubulogenesis.

### 4.4 Evaluation of organic transport function in generated tubules

The next objective of the thesis was to examine the function of the HK-2 cell-derived renal tubules. One of the main functions of the renal proximal tubule is the transport of potentially toxic organic anions such as metabolic wastes, drugs, and pollutants from the blood to the urine. To assess if this function was retained in the in vitro tubulogenesis model, generated tubules were incubated with the fluorescent organic anion, Lucifer yellow (LY). The lithium salt form of LY (Sigma) was used which is a water soluble dye with maximum excitation/emission at 428/536 nm and a molecular weight of 457.24.

Proximal tubule cells transport LY intracellularly and then secrete the dye into their lumens. This was shown by the study by Masereeuw et al. that used confocal microscopy to visualize the uptake of LY into killifish proximal tubule cells and the secretion into the tubular lumen. They were able to demonstrate by the addition of a variety of inhibitors, that LY is initially transported by the sodium-
Figure 4.4.1. Organic anion transport in tubular structures. Confocal images show tubular structures before and after 1 hour incubation with the fluorescent organic anion dye, Lucifer yellow. Scale bar, 100 μm.
dependent organic anion transporter and then secreted into the lumen through the sodium-independent organic anion transporter. To determine if the generated *in vitro* tubules in this study retained the same function, a fluorescent transport assay was developed.

Figure 4.4.2. Schematic of transporter washout experiment. Samples were incubated with LY for 1 hour to allow for the dye to accumulate in the lumen. The LY-media was then replaced with dye-free medium. The schematic shows the resulting situations that could occur: 1) LY could diffuse out of the tubules through a cavity, signifying the structures are not polarized or contain a cavity, this would be assumed to be quick (top diagram) or 2) LY remains in the lumen and in the proximal tubule cells, demonstrating that LY is actively transported through the cells.

HK-2 cells were cultured in starPEG-MMP-heparin hydrogels as previously described in Section 4.1. After 4 weeks in culture, the standard medium was exchanged to DMEM-HAM’s F-12 phenol red free medium. Representative tubular structures in the samples were then imaged with confocal microscopy. Next, LY was added to the medium and incubated with the hydrogels for 1 hour. At the end of the incubation time, samples were reimaged with confocal microscopy. At the beginning of the study, when the samples were imaged in phenol red free media, the lumens of the structures were empty. They were even void from background staining from the fluorescent DNA stain, Hoechst, which can be seen in the hydrogel surrounding the structures (Fig. 4.4.1). This suggests that the Hoechst stain solution does not penetrate the lumens, and supports the belief that the proximal
tubule cells form closed polarized epithelial structures. After 1 hour of incubation, Lucifer yellow could be seen in the lumens of the generated tubule structures (Fig. 4.4.1).

To verify that LY was transported through the proximal tubule cells and not entering the lumens through simple diffusion, a wash out experiment was conducted. After 1 hour incubation with LY, medium with LY dye solution was removed, and replaced with dye free media. After 20 hours of incubation, it was again imaged by confocal microscopy as shown in the schematic in Fig. 4.4.2. After the 20 hour washout, LY dye remained in the lumen of the tubular figure, determined by the higher fluorescent intensity compared to the hydrogel (Fig. 4.4.3). LY was also co-localized with some of the proximal tubule cells (arrow in Fig. 4.4.3). This demonstrates that the fluorescent organic anion dye enters into the lumens of tubular structures through an active transport system and not through unspecific diffusion (Fig. 4.4.2). Therefore, the organic anion transporters are functional in the in vitro generated tubules.

Figure 4.4.3. Washout experiment with organic anion dye to determine function of transporters. Confocal image of a renal structure before and after 20 hours washout with LY-free media. Scale bar, 100 μm.
Results and Discussion

4.5 Prospective of starPEG-MMP-heparin hydrogels for primary PTEC tubulogenesis studies

In the previous sections, a hydrogel-based tubulogenesis model was developed, characterized, and applied using immortalized proximal tubule cell lines. However, immortalized cell lines often do not retain all functions of their primary cell counterparts. Therefore, the next aim of the thesis was to validate the established hydrogel model using primary human proximal tubule cells, which are presumed to be closer to the \textit{in vivo} state than immortalized cell lines. Primary proximal tubule epithelial cells (PTEC) are difficult to culture due to dedifferentiation, limited supply, expense, and low passage use\textsuperscript{92}. To test the hypothesis that heparin-based hydrogels could promote tubulogenesis of primary PTECs, single PTECs were embedded in degradable starPEG-MMP-heparin hydrogels and non-degradable starPEG-heparin hydrogels. The same conditions were used as for HK-2 cells, with 50,000 cells seeded per gel and utilizing hydrogels with a storage modulus of \(~500\) Pa. The only difference between the cultures was the medium, which only had 0.5\% FBS and additional supplements that are described in detail in the Materials and Methods, Section 3.2.2. Primary PTEC were cultured in the hydrogels for 4 or 5 weeks. Phase contrast images were taken weekly to monitor primary PTEC morphogenesis.

By day 7 in starPEG-MMP-heparin hydrogels, single PTECs had formed small spheroids that expanded throughout the culture. Several spheroids formed a distinct epithelium by day 21 and day 28 and a few spheroids elongated and formed tubular structures, while a large majority of spheroids were small (less than 100 \(\mu\)M) (Figure 4.5.1). To investigate the polarization of the epithelial structures, samples were IF stained for the basolateral markers, \(\beta\)-catenin and \(\text{Na}^+/\text{K}^+\) ATPase, and the apical tight junction protein, ZO-1 (Figure 4.5.2).

![Figure 4.5.1. Morphogenesis of primary PTEC cultured in starPEG-MMP-heparin hydrogels.](image)

Representative phase contrast images of primary human proximal tubule cells from single cells to polarized renal structures throughout a 4 week culture. Scale bar, 500 \(\mu\)M.
Primary PTEC derived tubular structures expressed β-catenin, a cell-cell adhesion and gene transcription regulator, along the basolateral membrane (Fig. 4.5.2A). β-catenin is a subunit of the cadherin complex where it coordinates cell-cell adhesion and also regulates gene transcription by the Wnt signaling pathway. The structures also expressed the basolateral marker Na$^+/K^+$ ATPase, also known as sodium-potassium pump, which is an transmembrane enzyme fueled by ATP and responsible for pumping sodium out of cells and potassium into cells (Fig. 4.5.2B). The tight junction protein, (ZO-1), was also expressed on the apical membrane, signifying that tight junctions are present between the cells. These results verify that primary PTEC derived tubule-like structures are fully polarized. The majority of tubule-like structures had completely clear lumens as in the representative images in Fig. 4.5.2, suggesting that the structures are mature and functional.

To prove that primary PTEC retain their differentiated phenotypes while cultured in the hydrogels, the structures were stained for the proximal tubule marker, lotus tetragonolobus lectin (LTL). Primary PTEC in tubule-like structures expressed LTL, demonstrating that primary PTEC preserve their differentiated phenotypes after 5 weeks in culture in starPEG-MMP-heparin hydrogels. It should also be noted that not all structures in degradable starPEG-MMP-heparin hydrogels formed tubular structures by the end of 4 or 5 weeks and some remained as large spheroids (>100 μm). It is presumed that with more time these large spheroids would also elongate and form tubular structures. Also, by the end of the study there were a large percent of small spheroids (<100 μm). Future experiments will need to be done to explore this detail and increase the percent of tubular structures that are formed. It is possible that the culture conditions need to be optimized for primary PTEC, as they were previously optimized with the immortalized HK-2 cell line which could have different proliferation rates leading to differences in tubulogenesis. The difference in serum concentration between the two culture systems (0.5% FBS vs. 10% FBS) could be another reason for the lower efficiency of tubular formation with primary PTECs. It was previously shown in this thesis in Fig. 4.3.1 that higher concentrations of FBS led to increased metabolic activity and tubulogenesis of HK-2 cells, supporting this theory.

Primary PTEC were also cultured in non-degradable starPEG-heparin hydrogels in parallel. In non-degradable starPEG-heparin hydrogels, the majority of PTECs formed smaller spheroids (<100 μm) compared to cells in the degradable starPEG-MMP-heparin hydrogels. Some spheroids in the non-degradable starPEG-heparin hydrogels were fully or partially polarized which could be seen with β-
catenin and ZO-1 IF staining (Fig. 4.5.3). The polarized spheroids often had clear lumens similar to the tubule structures seen in the degradable starPEG-MMP-heparin hydrogels. However, some spheroids remained smaller and unpolarized, which can be seen in the zoomed out image in Fig. 4.5.3. These spheroids did not form lumens by week 4. It is possible that the unpolarized spheroids needed a longer time in culture to form organized epithelia with clear lumens.

Figure 4.5.2. Polarization of primary PTEC-derived tubules in starPEG-MMP-heparin hydrogels. (A) Confocal images of a tubular-like structure after 5 weeks of culture. Zoomed in images show β-catenin and the tight junction protein, ZO-1. Scale bar, 100 μm. (B) Confocal images of a tubular-like structure after 5 weeks in culture showing lotus tetragonolobus lectin (LTL) and Na+/K+ ATPase. Scale bar, 100 μm.

With the established degradable heparin-based hydrogel model, human primary PTECs were stimulated to undergo tubulogenesis under low serum (0.5% FBS) conditions. Previous tubulogenesis studies using primary human PTECs required the addition of hepatocyte growth factor (HGF) or high serum concentrations to promote tubulogenesis. Alternatively, the only growth factor added to cultures of PTECs in starPEG-MMP-heparin hydrogels was 20 ng/ml EGF and traces of a range of growth factors found in 0.5% FBS. Miya et al. found that even with the addition of 1,000 ng/ml of HGF, only about 60% of colonies formed tubular structures after 5 days in a mixture of Matrigel/collagen I. After 7 days in culture, the HGF-induced tubular structures were small with a diameter less than 10 μm, much lower than the human physiological size (41.5 ± 6.2 μm). In addition, TEM micrographs showed that the cells were shorter than PTECs in vivo (< 2
μm in Matrigel/collagen vs. ~8 μm in vivo\textsuperscript{57,135}. However, it is possible that, if the culture was kept longer, the structures would have grown larger.

Figure 4.5.3. Primary PTECs form spheroids in non-degradable starPEG-heparin hydrogels, similar to HK-2 cells. Confocal images of primary human PTECs after 4 weeks in culture. Scale bars, 100 μm.

Importantly, all previous 3D primary PTEC tubulogenesis models were cultured in Matrigel, collagen, or a mixture of Matrigel/collagen, which do not allow for tuning of the matrix to study tubulogenesis or for disease modeling\textsuperscript{57,94,95}. They also have batch to batch variation, leading to issues in reproducibility. To the best of our knowledge, this is the first model where human primary PTECs can be monitored from single cells to physiologically sized tubule structures in a fully controlled and tunable 3D matrix.
4.6 Potential of starPEG-MMP-heparin hydrogels as an *in vitro* nephrotoxicity model

The kidneys, in particular the proximal tubule cells, are massively affected by drug toxicity\textsuperscript{168,169}. Current nephrotoxicity assays rely on 2D cell culture and animal models\textsuperscript{65}. However, only a limited number of animal experiments accurately predict the human response to drugs\textsuperscript{8,9} and cells grown on conventional 2D tissue culture substrates lack the cell-cell and cell-matrix interactions that are crucial in maintaining cell phenotype and function\textsuperscript{10}. As a consequence, adverse drug reactions are often not found until human clinical trials. As an alternative to 2D systems, animal models provide a physiological 3D environment, but often cannot predict the response of the human kidney due to interspecies differences. Therefore, there is a critical need for more reliable and representative human nephrotoxicity assays which accurately recapitulate the human 3D structure and function of proximal tubules in a robust, tunable matrix that can be easily analyzed in throughput screenings for toxicity.

For these reasons, the established hydrogel-based tubulogenesis model was evaluated for its efficacy as a nephrotoxicity assay. Cisplatin, a chemotherapeutic drug known to be highly nephrotoxic, was chosen for the studies. Cisplatin has been shown to be actively transported into proximal tubule cells, where it leads to cell injury and apoptosis\textsuperscript{74,75}. HK-2 cells were embedded in starPEG-MMP-heparin hydrogels and cultured under standard conditions for 4 weeks to allow the cells to form branched structures as seen in Fig. 4.2.2C. The medium was then removed and replaced with serum-free DMEM/F-12 medium containing 0 to 100 μM cisplatin and incubated for 48 hours.

*Figure 4.6.1. Morphology of tubular structures before and after cisplatin treatment.* Samples were incubated with a range of cisplatin from 0-100 μM for 48 hour. Scale bar, 100 μm.
The architecture of the tubular structures was markedly altered after incubation with the nephrotoxic cisplatin. After incubation with 50 µM of cisplatin, the architecture of some tubular structures started to deteriorate which can be seen in Fig. 4.6.1. To investigate this further, and also determine the amount of apoptotic cells in the culture, samples were stained for F-actin and cleaved caspase-3, a marker for apoptosis. The control samples show clear actin staining around the borders of the renal structures, as expected (Fig. 4.6.2). However, with the addition of cisplatin the presence of actin became less pronounced and with the highest concentration of 100 µM cisplatin, only small sections of actin were remaining (Fig. 4.6.2). Importantly, there was a dose-dependent response in apoptotic cells, which agrees with established mouse models of nephrotoxicity that reported increased apoptosis in proximal tubule cells\(^{75}\) (Fig. 4.6.2).

It has previously been reported that at low concentrations cisplatin induces apoptosis in primary mouse proximal tubular cells whereas high concentrations lead to necrosis\(^{80}\). For this reason, the amount of necrosis in the cultures was also examined by measuring the lactate dehydrogenase (LDH) release in the supernatant. LDH is an enzyme released by cells following injury to the plasma membrane and can be used to quantify necrotic cells\(^{133}\). After a 48 hour incubation with cisplatin, LDH was measured in the supernatant. The metabolic activity of the cells was also analyzed.

![Figure 4.6.2. Dose-dependent apoptosis response to nephrotoxins.](image)

A decrease in metabolic activity and an increase in LDH release was observed with increasing concentrations of cisplatin (Fig. 4.6.3.). These results show that cisplatin has a dose-dependent effect on the tubular structures. From the metabolic activity results the EC\(_ {50}\) value for the hydrogel-based tubulogenesis model is estimated to be \(~27~\mu M\). In contrast, conventional 2D cultures with kidney
cell lines often require dosages that are orders of magnitude higher. For example, Astashkina et al. found that the kidney cell lines HEK and LLC-PK1 have a cisplatin EC\textsubscript{50} value of 2.7 mM and 2.0 mM, respectively\textsuperscript{170}. In contrast, in our hydrogel-based model, HK-2 derived tubular structures have an EC\textsubscript{50} that is close to the value found by Li et al. for primary human proximal tubule cells (39.57 \textmu M, determined by cell viability)\textsuperscript{171}. Therefore, the established tubule model is a better representation of human primary proximal tubule cells than other renal cell lines.

Additionally, the established hydrogel-based tubule model was compared to animal nephrotoxicity models. Cisplatin dosages were recalculated to our model using the average weight per experimental well which was found by Bray et al. to be 35.38 mg per gel and 1 g of solution, making a total of 1.03538 g\textsuperscript{101}. Established mouse models of nephrotoxicity commonly use a dose of 20 mg/kg cisplatin body weight, which results in an increased apoptosis in proximal tubule cells\textsuperscript{75,172}. In comparison, our \textit{in vitro} tubulogenesis model after incubation with 20 mg/kg (69 \textmu M cisplatin calculated to our system), shows an increase in apoptosis that can be seen in the expression of cleaved caspase-3 IF, as well as a decrease of metabolic activity and increase in cytotoxicity compared to controls (Fig. 4.6.2 and Fig. 4.6.3). Therefore, our \textit{in vitro} hydrogel-based model could potentially be used in the future as an alternative to animal nephrotoxicity models.

![Metabolic Activity and Cytotoxicity Graph](image)

**Figure 4.6.3.** Cisplatin-induced dose response on metabolic activity and LDH release. Metabolic activity and cytotoxicity (LDH release) of tubular structures after 48h treatment with cisplatin (Mean of pooled experiments \pm s.e.m., n = 2-4, experiments performed in triplicate).
Metabolic activity, apoptosis, and necrosis are common markers for drug-induced injury, however, recent interest has gone into the exploration of renal biomarkers that can detect the early onset of injury and disease\textsuperscript{64,173}. To test the sensitivity of the generated tubular structures, the expression of the \textit{in vivo} proximal tubule specific renal injury biomarker, KIM-1, was then investigated after incubation with cisplatin. Immunofluorescence staining revealed that KIM-1 was upregulated in tubular structures after treatment with 50 \(\mu\)M cisplatin (Fig. 4.6.4). KIM-1 was also expressed at lower levels in 25 \(\mu\)M treated samples, signifying a dose dependency of its expression. It should also be noted that some renal structures treated with 25 \(\mu\)M cisplatin had KIM-1 levels similar to the untreated control (Fig. 4.6.4), suggesting that the critical concentration that induces KIM-1 is between 25 and 50 \(\mu\)M.

To validate these results, the experiment was repeated with primary human PTECs using 25 and 50 \(\mu\)M of cisplatin. The same conditions were used as for the HK-2 cell nephrotoxicity experiments, with the exception that PTEC media was used instead of HK-2 media. The primary PTEC derived tubular structures expressed KIM-1 after incubation with 50 \(\mu\)M cisplatin (Fig. 4.6.5). When the PTEC derived tubular structures were incubated with 25 \(\mu\)M of cisplatin they showed a variable expression of KIM-1. Thus, the primary PTC derived structures confirm the nephrotoxicity results from our previous HK-2 cell study and show potential as a model for personalized nephrotoxicity.
Figure 4.6.4. Expression of the biomarker KIM-1 after cisplatin treatment. Confocal images of kidney injury molecule-1, KIM-1, expression in HK-2 cell derived tubular structures after 48 hour cisplatin treatment. Scale bar, 75 µm.

The established model was then compared to doses used in the human clinic. The highest dose used on humans is 100 mg/m². This concentration was recalculated to our system using the $K_m$ factor method proposed by Reagan-Shaw et al. Specifically, the dose was divided by the human $K_m$ factor to get a concentration in mg/kg. Then the dose of drug needed per well was found using the average weight in each well, 1.03538 g as stated earlier. The value was then converted to moles using the molecular weight of cisplatin and finally, the molarity was found in 1 ml of media. By applying this method, the highest human dose of cisplatin, 100 mg/m², would be ≈ 9 µM. Although this cisplatin concentration is perhaps too low to show an increase in apoptosis in our model, it does show a
decrease in metabolic activity and an increase in LDH release (Fig. 4.6.3). Hence, our hydrogel-based \textit{in vitro} tubulogenesis model is sensitive to nephrotoxin concentrations that affect the human kidney. Altogether, this established model could make a large impact on the field of nephrotoxicity by providing more reliable preclinical assays and limiting the need for animal models.

Figure 4.6.5. Cisplatin-induced expression of KIM-1 in primary human PTEC-derived tubular structures. Primary human PTECs were cultured in starPEG-MMP-heparin hydrogels for 4 weeks and then subsequently incubated with cisplatin for 48 hours. Confocal micrographs show IF staining of the biomarker, KIM-1, after treatment with cisplatin. Scale bar, 50 μm.
Chapter 5

General Discussion

5.1 Summary and conclusions

Due to the rising prevalence of chronic kidney disease and end stage renal disease, scientists have begun to research alternative renal regenerative therapies. In order to discover new therapies, an accurate model of the human kidney is required. The ideal model would contain the complex functions of the human kidney in a tunable and easily observable system. Currently, the majority of preclinical trials are performed with animal models, which often contain interspecies differences. An in vitro renal model could circumvent these issues by providing a system where human cells can be used and the response to treatment could be visualized in real time, rather than sacrificing animals at predetermined time points. In this way, the strength and duration of a treatment could be better evaluated. A 3D in vitro model would also be a better representation of the physiological environment compared to 2D cell culture models, while still allowing simple cell modification such as gene editing. Although there have been many advances in this field in the past few years, a clinically relevant 3D model that retains the functions and anatomy of the mature human kidney in a tunable system has not yet been established\textsuperscript{20,176}.

The aim of this thesis was to create an in vitro 3D renal tubulogenesis model using the tunable biohybrid starPEG-heparin hydrogel platform. Hydrogels with a range of mechanical properties and compositions were evaluated to determine the optimal conditions to promote renal tubulogenesis. The results revealed that soft hydrogels composed of heparin and MMP cleavable peptides (starPEG-MMP-heparin hydrogels) were the only condition to produce tubule structures. The tubules displayed polarization markers, extracellular matrix components, and organic anion transport function of the human renal tubule.

Remarkably, in starPEG-heparin hydrogels (no MMP cleavable peptide), PTECs formed polarized spheroids, whereas in degradable starPEG-MMP-starPEG hydrogels (no heparin), the cells formed unorganized colonies. From these results, two main conclusions were determined: 1) heparin plays a role in the organization of PTECs in the presented 3D model and 2) the combination of heparin and
a cleavable matrix are crucial for the formation of tubular structures in the hydrogel culture. Interestingly, heparan sulfate, which has similar characteristics to heparin, is the main GAG found in human kidney and has been reported to facilitate lumen formation in organotypic cultures of embryonic lung epithelial cells\textsuperscript{177,178}. To the best of our knowledge, this is the first time that heparin has been incorporated into a hydrogel for use as a proximal tubule model. These results also indicate that immortalized proximal tubule cell lines (HK-2 cells) have the ability to form physiologically relevant proximal tubule structures, possibly limiting the dependence on less assessable primary proximal tubule cells.

To probe the ability of the hydrogel-based system as a model for renal tubulogenesis, experiments were performed with pro- or anti-tubulogenic soluble factors supplemented in the culture. A serum substitution study to determine the importance of growth factors in serum was also performed. Results from these studies showed that growth factors are necessary for tubulogenesis and the generation of tubule structures can be enhanced by increasing concentrations of single factors. In contrast, when a MMP inhibitor was added to the cultures, tubulogenesis was inhibited. Thus, the findings revealed that tubulogenesis could be modulated by adding soluble cues to the culture medium and to the hydrogels themselves. In overall, these results suggest that the hydrogel-based model can be used for studying renal tubulogenesis, potentially for regenerative medicine applications.

As a proof of principle, the hydrogel-based model was then applied to primary PTECs. Single primary PTECs extracted from healthy human kidneys were embedded in the hydrogels. Findings showed that human primary PTEC formed polarized epithelial structures with clear lumens in starPEG-MMP-heparin hydrogels. In addition, the renal structures also possessed the proximal tubule specific lectin, \textit{lotus tetragonolobus} (LTL), confirming that the PTECs retain their differentiated phenotype and generate proximal tubule-like structures. Remarkably, these polarized structures formed in culture conditions of only 0.5% FBS.

As far as we are aware, this is the first time that human primary PTECs have been stimulated to undergo tubulogenesis \textit{ex vivo} without the addition of HGF or high serum concentrations in a synthetic matrix. Results from the primary PTEC study indicate that renal tubule cells have an inherent ability to reconstruct tubule-like structures and therefore, could have an important use in
renal regenerative medicine. The source of *in vivo* tubule regeneration is a controversial topic with different modes of regeneration believed to be active after injury; they include dedifferentiation of PTECs, compensatory hyperplasia, and a resident pool of progenitors/stem cells among PTECs\textsuperscript{179,180}. The established renal tubulogenesis model could be used to further study these mechanisms.

The next aim of the study was to evaluate the efficacy of the hydrogel-based renal model as a nephrotoxicity assay. The potent chemotherapy drug cisplatin, a known nephrotoxin, was incubated with the renal tubule model. The tubular structures showed a dose-dependent drug response and the injured tubular structures expressed the early *in vivo* proximal tubule injury biomarker, kidney injury molecule-1 (KIM-1). Remarkably, the generated tubules were responsive to drug concentrations on the same range as concentrations used in the human clinic. Therefore, the developed renal tubulogenesis model recapitulates a similar nephrotoxic response of the human kidney.

Altogether, in this thesis, we successfully developed and characterized a renal tubulogenesis model based on starPEG-MMP-heparin hydrogels. To the best of our knowledge, this is the first system where human renal tubulogenesis can be monitored *ex vivo* from single cells to physiologically sized tubule structures in a 3D tunable matrix. This model can be used to modulate tubulogenesis by adjusting hydrogel mechanics, growth factor signaling, and the presence of insoluble cues (such as adhesion peptides), potentially providing applications for regenerative medicine or disease modeling. Additionally, the model could be used for drug toxicity tests as an alternative to animal models. The ability of immortalized PTEC lines to create human-like structures opens options for high throughput assays, whereas the results with primary PTEC demonstrate that the model could potentially be used for personalized medicine. In conclusion, a versatile 3D renal tubulogenesis model was successfully established using tunable, degradable starPEG-MMP-heparin hydrogels and shows promise in a range of applications.

### 5.2 Future perspectives

The established hydrogel-based renal tubulogenesis model is a versatile tool for studying renal tubulogenesis. However, it does contain some limitations and ways where it can be approved upon. This section will briefly comment on these limitations and then how the model can be further utilized for future applications.
An important finding of the thesis is the discovery of heparin’s contribution to proximal tubule cell polarization in 3D cultures. As it was previously mentioned in Section 4.2.2, there are various reasons that could contribute to this occurrence such as the stabilization of soluble factors or the anchoring of ECM. To better understand the role of heparin, it would be interesting to embed human proximal tubule cells in other GAG-based hydrogels. For example, hydrogels could potentially be formed with chondroitin sulfate, a glycosaminoglycan consisting of repeating disaccharide units containing N-Acetylgalactosamines rather than the N-acetylglicosamines in heparin. Chondroitin sulfate could be used to form starPEG-chondroitin sulfate hydrogels that are similar to starPEG-heparin hydrogels. In this way, specific characteristics of heparin such as charge, size, and structure could be investigated to determine their influence on renal tubulogenesis.

Another GAG-based hydrogel that could give important insights into the mechanism behind heparin is hyaluronan, a nonsulfated glycosaminoglycan. Embedding proximal tubule cells in engineered hydrogels constructed of starPEG and hyaluronan would show how the lack of sulfate groups affects renal tubulogenesis. If proximal tubule cells do not form tubular structures in starPEG-hyaluronan hydrogels, it would highly suggest that the sulfate groups, and hence, the charge of the heparin is crucial in promoting renal tubulogenesis. This theory could be probed even further by embedding proximal tubule cells in starPEG-MMP-heparin hydrogels with various degrees of desulfation. This could be valuable knowledge for kidney development and renal regenerative medicine studies, as well as improve upon the established renal tubulogenesis model.

Another finding from the thesis was the influence of FBS and growth factors, specifically HGF on renal tubulogenesis. It was shown that immortalized proximal tubule cells (HK-2) do not form tubular structures when incubated with the commercially available serum substitute, panexin NTA (Fig. 4.3.2), instead of the standard FBS. It would be interesting to see if this effect could be reversed, particularly if HGF and/or additional growth factors could be used in combination with the serum substitute to promote renal tubulogenesis that occurs with 10% FBS. If this could be achieved, it would present a simplistic model where individual soluble factors can be examined without the presence of the complex FBS.
The established renal tubulogenesis model could also be used to further investigate the individual MMPs that influence renal tubulogenesis. As it was shown with the addition of a broadband MMP inhibitor, along with other published studies, MMPs are needed for renal tubulogenesis. However, in the established starPEG-heparin hydrogel renal tubulogenesis model, the distinct MMPs necessary are unclear. A wide range of MMP inhibitors have been created that can impede the function of two MMPs or even a single MMP. By adding these inhibitors to the established renal tubulogenesis model, the role of specific MMPs can be determined. Understanding the MMPs that are involved in proximal tubulogenesis is important for the creation of new regenerative therapies, where MMPs could potentially be used as a treatment.

It was shown that the organic anion dye, LY, could be transported from the basal side of the renal structures (in cell culture medium) to the apical side of the renal structures (lumen). Although this data suggests that LY is being transported into the cells through organic anion transporters, it presents an indirect proof. In order to directly prove that the organic anion transporters are active in the movement of LY, the commercially available organic anion transporter (OAT) inhibitor, probenecid could be introduced to the cultures. If the addition of probenecid, decreased the amount of LY that was displayed in the lumen of renal tubule structures, it would prove that the OATs are actively transporting LY through the cells.

The use of other fluorescently labeled proteins with various molecular weights could be used to further test the tissue-specific barrier functions of the renal tubule structures and verify the organic transporters. For example, the polysaccharide dextran is commercially available with molecular weights ranging from 3-70 kDa and can be conjugated to fluorescein isothiocyanate (FITC) or rhodamine for visualization. Freedman et al. found that rhodamine-dextran (MW 10 kDa) localized to tubular lumens of the majority of renal organoids and Takasato et al. saw proximal tubule regions of organoids endocytosed dextran-Alexa488. This effect could be markedly reduced by the addition of latrunculin B which inhibits actin polymerization and endocytosis. This approach could easily be repeated in our model using the transporter assay that was constructed to test LY transport. In addition, to verify that the proximal tubule cells form tight junctions in the tubular structures, inulin-FITC, a low molecular weight (4.5 kDa) compound that is neither reabsorbed nor secreted by in vivo renal tubules, could also be added to the cultures. If no inulin-
FITC accumulated in the lumen of the generated renal tubules, it would verify that the proximal tubule cells form fully enclosed tubular structures that consist of a tight monolayer of cells.

Another way the tissue specific functions of the generated renal tubule structures could be examined is by their response to other non-renal toxins. For example the renal tubulogenesis model could be incubated with the liver toxin, D-galactosamine, which causes apoptosis and necrosis in hepatocytes \textit{in vivo} \cite{185,186}. If the treated proximal tubule cells show a similar response to the untreated control, it would prove that the proximal tubule cells retain tissue specific function.

The hydrogel model was applied to human primary proximal tubule cells where they formed polarized renal structures. Some of these structures elongated and formed tubule like structures. However, further studies should aim to increase the amount of tubule structures that are formed from primary proximal tubule cells. This could be done by changing cell concentrations and hydrogel stiffness. Also, the addition of growth factors such as HGF, EGF, and FGF-2 could be implemented in the cultures to increase the rate of tubulogenesis.

In this thesis, it was shown that the established hydrogel-based renal tubulogenesis model could be used as a nephrotoxicity assay through the results with cisplatin incubation. To verify this further, other nephrotoxins could be applied to the system using the same nephrotoxicity parameters established in this thesis. In addition to nephrotoxicity applications, the hydrogel-based model can also be used for disease modeling. For example, the mechanical properties of the hydrogel can be adjusted to simulate a fibrotic state that is representative of end stage renal disease.

It would also be interesting to explore the cisplatin-induced renal tubule injury model further to determine if the injury could be recovered. This could be done by incubating the generated tubules with cisplatin for a specific time point, then replacing the medium with normal cisplatin-free medium and keeping the samples in culture. The injury of the proximal tubules could be monitored over time to determine if they can recover similar to \textit{in vivo}. If this would be possible, the model could be used as an \textit{in vitro} acute renal injury model.

One of the main limitations of the model is that is a simplistic view of the nephron and does not contain the full complexity of the kidney. In the \textit{in vivo} environment, proximal tubules are adjacent to
peritubular capillaries with which they are constantly exchanging molecules through reabsorption and secretion (Fig. 2.1.1). To replicate this in the established hydrogel system, renal proximal tubule cells could be co-cultured with endothelial cells in starPEG-MMP-heparin hydrogels. Previous studies have already shown that endothelial cells can form blood vessels in the \textit{in situ} starPEG-MMP-heparin hydrogels\textsuperscript{100}. Therefore, it would be interesting to see if blood vessels could form parallel to the formation of the renal tubules, if the rate of tubulogenesis could be increased, and if the structures would arrange together.

This proposed co-culture model could be valuable to have a more morphologically correct model of the proximal tubule environment. However, there would still remain a lack of blood supply, limiting the exchange of molecules between the two structures. To address this concern, the hydrogel-based co-culture system could be grafted to a chicken chorioallantoic membrane (CAM)\textsuperscript{187,188}. This would potentially provide a source of blood to the system. An alternative way to assess the starPEG-heparin hydrogel-based renal tubulogenesis model \textit{in vivo} is implantation of the hydrogel under the kidney capsule\textsuperscript{189}. This method could provide vascularization to the system through the kidneys own blood vessels. Finally, the physiological function of the proximal tubules could be evaluated by injecting fluorescently conjugated proteins into the blood system of the animal host to see if they accumulate in the generated implanted tubule structures.

Additionally, if the 3D renal model could be used in a high-throughput design it would broaden its applicability as a preclinical model for drug screening or regenerative medicine studies. This could potentially be done using a 3D printer to print the hydrogels more efficiently or optimizing the model for use in 96 well plates to increase the number of factors that can be examined in one experiment. Future studies should apply these methods to the starPEG-MMP-heparin hydrogel renal tubulogenesis model to demonstrate the efficacy of the model for high throughput screening. In conclusion, we have established a hydrogel-based renal tubulogenesis model that can be used for several different applications.
Appendix

Chapter 6: Supplementary materials and methods

6.1 Materials for peptide synthesis
All solvents and reagents for peptide synthesis were purchased from IRIS Biotech GmbH (Marktredwitz, Germany). Tris (2-carboxyethyl) phosphine (TCEP) was purchased from Sigma-Aldrich Company (St. Louis, USA). Four-armed maleimide terminated polyethylene glycol (starPEG-mal) (MW = 10.0x10^3; PDI = 1.08) and four-armed thiol terminated polyethylene glycol (starPEG) was purchased from JenKem Technology USA Inc. (Allen, USA). All the reagents were used without preliminary purification.

6.2 Methods for peptide and conjugate synthesis
All peptide synthesis and purification of the MMP cleavable peptide and the starPEG-MMP conjugate was performed by Dr. Mikhail Tsurkan. Milauscha Grimmer performed all synthesis and purification of heparin-maleimide conjugates.

6.2.1 Peptide syntheses and purification
The MMP-cleavable Ac-GGPQGIWQGGCG-NH2 (MMP) peptide was synthesized by solid-phase methods using an Activo P14 (Activotec, Cambridge UK) peptide synthesizer and standard Fmoc-chemistry. Specifically, a 4 mmol scale protocol with a C-terminal capping protection strategy by amide was used. Amino acid activation was achieved by O-(benzotriazol-1-yl)-N, N, N', N'-tetramethyluronium tetrafluoroborate (TBTU), and 1-hydroxybenzotriazole (HOBT) in DMF with diisopropylethylamine (DIPEA) as a base. Deprotection of amino acid side chains and cleavage from the resin was performed by reaction with a mixture of trifluoroacetic acid (TFA) (87, 5% v/v), phenol (5% v/v), tri-isopropyl silane (TIPS) (2.5% v/v), and water (5% v/v) for 2.5 hours at room temperature. The crude peptides were then precipitated in anhydrous diethyl ether, collected by vacuum filtration, and dried under vacuum. Final purification was achieved by preparative reversed-phase high performance liquid chromatography (HPLC). All peptide purification was performed by HPLC on a preparative reversed-phase Phenomenex Lune Prep C-18 column (10μM particle size,
A linear gradient of water/acetonitrile containing 0.1 % (v/v) trifluoroacetic acid was used as the mobile phase. The HPLC separation runs were performed over 30 min using the flow rate of 10 ml/min and the monitoring wavelengths were set to a wavelength range of 210-278 nm. A two-pump system (1200 Series; Agilent Technologies, Santa Clara, USA) equipped with an UV/Vis diode array detector/spectrophotometer and a 1 cm path length cell was used. The collected peptide was lyophilized, and its purity was verified by analytical HPLC and electrospray ionization mass spectrometry (Agilent Technologies 6230 TOF LC/MS spectrometer, Applied Biosystems, USA).

6.2.2 Synthesis and purification of cysteine-terminated PEG-(MMP)$_4$

StarPEG-MMP conjugate was synthesized as previously described$^{29}$. In brief, 100 mg of four arm star-shaped, maleimide-terminated PEG-(Maleimide)$_4$ (Mw=10000) was dissolved in 1 ml of water and mixed with 61 mg of MMP peptide (5% excess) which was dissolved in 0.5 ml of ddH$_2$O. Next, 0.5 ml of Phosphate buffer pH 7.4 was added. The pH of the reaction mixture was adjusted to pH 7.5-8 by 1M NaOH. The reaction was run for 5 hours under N$_2$ atmosphere, and the completion of the reaction was followed by HPLC. In order to remove StBu protection group, a five times molar excess (to the peptide) TCEP solution (pH 7-8) was added to the reaction mixture and was stirred at room temperature for several hour. Next, the reaction mixture was evaporated till dry, dissolved in water and purified by preparative HPLC. The product was collected from the HPLC, mixed with 0.1 ml of 1M HCl and freeze-dried for greater than 24 hours. The formed white powder was then stored at -20°C.

6.2.3 Synthesis and purification of heparin-maleimide conjugates

All the heparin maleimide conjugates were synthesized as previously described$^{29,32}$. Briefly, Threefold molar excess of EDC and 1.5 fold molar excess of sNHS (referring to the maleimide amine) were added to a solution of 0.5 g of heparin dissolved in 3 ml of ultrapure H2O. The reaction mixtures were kept at 4 °C for 20 min. Next, N-(2-aminoethyl) maleimide trifluoroacetate salt dissolved in ddH2O (at 5 °C) was added to the reaction mixtures and stirred overnight at room temperature. The amount of maleimide amine (5% excess to the theoretical amount) was 57.3 mg for HM6 and for 76.5 mg HM8 conjugate which correspond to six and eight maleimide molecules per heparin correspondently. The conjugates were purified by dialysis (membrane with 8kDa molecular weight
cut-off) against 1L of 1M sodium chloride three times followed by dialysis against 1L of water five times in order to remove any unreacted maleimide amine. The products were then freeze-dried for at least 24 hours and stored at -20 °C. The purity and reactivity of the formed conjugates were controlled by HPSEC (1100 Series; Agilent Technologies, Santa Clara, USA). For these studies, heparin was functionalized with maleimide groups at a molar ratio of 1:8.
Figure 7.1. Comparison of the ultrastructure between in vivo and in vitro renal structures. (A) TEM micrograph of a descending thin limb in a rat kidney. Arrows represent tight junctions. Scale bar, ~6 μm using X3750 parameters. Adapted from Pannabecker190. (B) TEM micrograph of HK-2 cell-derived structure cultured in degradable starPEG-MMP-heparin hydrogels. HK-2 cells were cultured for 4 weeks and then fixed and processed for TEM imaging. Scale bar, 10 μm. The reader is referred to Junqueira’s Basic Histology Text & Atlas for a TEM micrograph of a thin limb of the loop of Henle in a human kidney22.
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## Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>2D</td>
<td>Two-dimensional</td>
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<tr>
<td>3D</td>
<td>Three-dimensional</td>
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<tr>
<td>AKI</td>
<td>acute kidney injury</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BUN</td>
<td>blood urea nitrogen</td>
</tr>
<tr>
<td>CAM</td>
<td>chicken chorioallantoic membrane</td>
</tr>
<tr>
<td>ciPTEC</td>
<td>conditionally immortalized proximal tubule epithelial cells</td>
</tr>
<tr>
<td>cisplatin</td>
<td>Cis-Diamineplatinum (II) (chemotherapeutic drug)</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified essential media</td>
</tr>
<tr>
<td>HK-2</td>
<td>immortalized human proximal tubular epithelial cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FCS/FBS</td>
<td>Fetal calf serum/fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein-isothiocyanate</td>
</tr>
<tr>
<td>GAGs</td>
<td>glycosaminoglycans</td>
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<td>GBM</td>
<td>glomerular basement membrane</td>
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<tr>
<td>HCS</td>
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<td>human embryonic kidney cells</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>kPa</td>
<td>kilopascal</td>
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<td>LTL</td>
<td><em>lotus tetragonolobus</em> lectin</td>
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<td>LY</td>
<td>Lucifer yellow (organic anion dye)</td>
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<td>MDCK</td>
<td>Madin-Darby canine kidney epithelial cells</td>
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<tr>
<td>MM</td>
<td>metanephric mesenchyme</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>MRP</td>
<td>multidrug resistance proteins</td>
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<td>PEG</td>
<td>poly (ethylene glycol)</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>penicillin/streptomycin</td>
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<td>PEG</td>
<td>poly (ethylene glycol)</td>
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<td>PTEC</td>
<td>proximal tubule epithelial cells</td>
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<td>OAT</td>
<td>organic anion transporter</td>
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<tr>
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<td>Arg-Gly-Asp</td>
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<tr>
<td>SCr</td>
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<tr>
<td>s.d.</td>
<td>standard deviation</td>
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<tr>
<td>s.e.m.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>sNHS</td>
<td>N-hydroxysulfo-succinimide</td>
</tr>
<tr>
<td>starPEG</td>
<td>four-armed thiol functionalized PEG</td>
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<td>four-armed maleimide terminated PEG</td>
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<td>starPEG-MMP</td>
<td>four-armed PEG end functionalized with MMP cleavable peptide</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
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<td>ureteric bud</td>
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<td>zonula occluden-1</td>
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Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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Meine Person betreffend erkläre ich hiermit, dass keine früheren erfolglosen Promotionsverfahren stattgefunden haben.

Ich erkenne die Promotionsordnung der Fakultät für Mathematik und Naturwissenschaften, Technische Universität Dresden an.

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