Development of a kidney-on-a-chip model for compound screening and transport studies

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Chapter 1

Introduction

Introduction

For safety determination of new drugs, animal studies are still required by the U.S. Food and Drug Administration (FDA) prior to clinical trials on humans [1]. Also, the European Medicines Agency (EMA) and the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) require animal studies before allowing the release of new drugs [2], [3]. However, the EMA recently published a document which includes opportunities for alternatives [2] and since 2013 animal testing is not allowed anymore in Europe in cosmetic products when known ingredients are used [4], [5]. The fact that more than 80% of the drugs which were tested in animal models failed during clinical trials in humans [6] supports the huge impetus to refine and replace animal testing, which is also known as the 3Rs: Replace, Reduce and Refine. The goal of this endeavor is replacing animal testing whenever possible, and reducing the number of animal tests. When strictly necessary, refining animal tests in order to use a minimum amount of experiments and animals is desirable [7]. The reasoning of using animals for drug testing goes back to the declaration of Helsinki 1964, which recommends animal testing when appropriate and when the welfare of animals is respected [8]. The benefits of animal testing are obvious: In animals, drugs can be tested in complete organisms. In several other types of drug testing, such as in cell cultures dishes, testing can only be performed on a single or on several cell types, depending on the culture-possibilities. Animals possess functioning vascular networks which supply the tested organ(s) with nutrients and oxygen. This also has the additional benefit of these vascular networks eliminating waste products. An example of a combination between animal testing and human tissue is when immunodeficient mice can be used for xenografting. Here, human tissue is implanted into an animal model which then has the possibility to vascularize the tissue. These models are known as patient-derived xenografts [9].

Nonetheless, considering the surprisingly huge number of clinical failures which could not have been predicted by animal testing, alternatives need to be investigated as Human cells sometimes respond quite differently to certain substances (medication or cosmetics) than animals cells [10]. Other important reasons why different testing possibilities should be considered are grounded in ethics: not only because of compassion for the animals used for testing but also for humans who participate in clinical trial studies and first patients that receive the new drug [11], [12].

For reducing animal testing primarily in the early stages of pre-clinical testing, 2D cell models have already been used for years [13]. The problem with these 2D models is not difficult to grasp: Cells are cultured on flat plastic or glass surfaces in an environment which does not resemble their natural environment very accurately. In a living organism, cells are encapsulated by a complex network of an extracellular matrix (ECM) in which they grow in close proximity to several other cell types [14].

In this thesis, a 3D *in vitro* model of the kidney will be developed, which should be able to play a big future role in not only the reduction and replacement of animal testing, but also to make medical compound testing more predictable for humans and more cost efficient. The model should be available for pharmaceutical, industry, and academic research to be utilized for studying nephrotoxicity, compound excretion, drug-drug interaction studies, disease modeling, and tests regarding the safety of cosmetics and chemicals.

The importance of the kidney during drug research

Kidney disease is a huge problem in our society which causes many deaths, mainly in hospitalized patients. Each year worldwide around 1.7 million people die of acute kidney injury (AKI) [15]. AKI is characterized as a rapid decrease of the kidney excretory function and urine production [16], [17]. AKI is commonly diagnosed by measuring the concentration of serum urea and creatinine, decreased urine output, or a combination of both to determine the glomerular filtration rate (GFR) [16]. However, the serum creatinine concentration only changes after already half of the GFR is gone, and often renal injury starts before GFR can be measured [16]. To overcome this problem, new biomarkers, such as such as albumin and total protein, neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule 1 (KIM-1), were discovered and are already used for diagnosis. [15], [16]. The use of such markers will eventually lead to an early treatment of AKI and will help to prevent severe cases [15]

AKI is mainly found in one of the four most important structures of the kidneys: the interstitium, the renal blood vessel system, the glomeruli, or the tubules [17]. In this thesis we will focus on the damage to the tubules which can be caused by two major factors: Drug-induced by exogenous and endogenous compounds, and renal ischemia (loss of perfusion) [17].

Around 30% of applied drugs for multiple target conditions relinquish the body unchanged through the kidneys [18] which are therefore vulnerable to (drug-induced) toxicity [19]. Approximately 20% of AKI is induced by drugs in community- and hospital-obtained occurrences. This percentage increases rapidly to more than 60% for older patients [20].To decrease this high rate of cases, compound studies of drug libraries as well as the interactions among drugs need to be studied before a drug is approved to enter the market [21]. Until now only a fraction of potentially nephrotoxic drug candidates are rejected because of nephrotoxicity in pre-clinical studies [21], [22]. Reasons are diverse as the toxicity usually is a result of a combination of factors which involve interaction of the organism with the drugs itself, drug metabolites, and drug–protein conjugates [21]. Elimination of drugs in the kidney is mainly facilitated by membrane transporters. When this pathway is restrained by improperly functioning transporters, drug-transporter interaction, or by drug-drug interaction compounds can accumulate in the cell cytoplasm leading to cellular and tubular damage [23], [24].

In other cases, AKI can be caused due to a comorbidity that disrupts renal perfusion, either because of pre-renal hypoperfusion (e.g. heart failure, hemorrhage) or post-renal obstruction (e.g. cancer, blood clot) [17], [25]. As a result of reduced renal perfusion, the kidney cells suffer from cell damage. Renal ischemia/reperfusion injury (rIRI) initiates a cellular response leading to cell damage, cell death, inflammation, and ultimately AKI [26] [27]. Therefore, assessment of the possibilities of compounds which act in a protecting way during the event of AKI should not be disregarded either.

The diversity of reasons causing AKI makes the requirements for future test platforms complex: Models are needed which need to be predictive for humans, but which also should be available in a high-throughput fashion to test this enormous number of compounds. Both of these conditions require a deeper understanding of the mechanisms of how the kidneys function in order to create a model that can predict the potential of drugs, or drug-drug combinations, to induce AKI.

The kidney

The kidneys are two bean-shaped organs which are found on the left and right side in the retroperitoneal space of the human body. They are responsible for filtering and cleaning the blood from toxic metabolites like urea, uric acid, as well as from (metabolized) drugs. All these compounds are eventually collected in the bladder and removed from the body as urine. Furthermore, the kidneys are responsible for maintaining the homeostasis of the extracellular fluid (pH, sodium, potassium, and calcium concentrations, osmotic pressure), for the regulation of the fluid circulation, and for the production of hormones. The functional units of the kidneys are called nephrons. Each kidney consists of around 1.2 million nephrons, of which each single nephron is able to produce urine as its end product (fig. 1A). Blood capillaries are covered by the Bowman's capsule which is the beginning of the tubular system of the kidneys (fig. 1B). In the glomerulus, blood is mainly filtered from blood cells and proteins by specialized cells called podocytes. In the tubular system behind the glomerulus, further excretion of toxic compounds from the interstitial fluid into the glomerular filtrate takes place. Also, reuptake of water, glucose, nutrients, and electrolytes is a process which happens in the tubular system. These processes make sure the filtrate is concentrated more and more into urine. The filtrate first enters the proximal tubules, then the loop of Henle which is followed by the distal tubule before it flows into the collecting duct and is excreted as urine.

The proximal tubules

Proximal tubules are the part of the nephron where drugs and metabolites are actively eliminated from the body [30]. This pivotal role makes them of high importance during drug development. The proximal tubules are represented by an polarized epithelial layer which functions as a passive and active filter membrane (fig. 2 A).

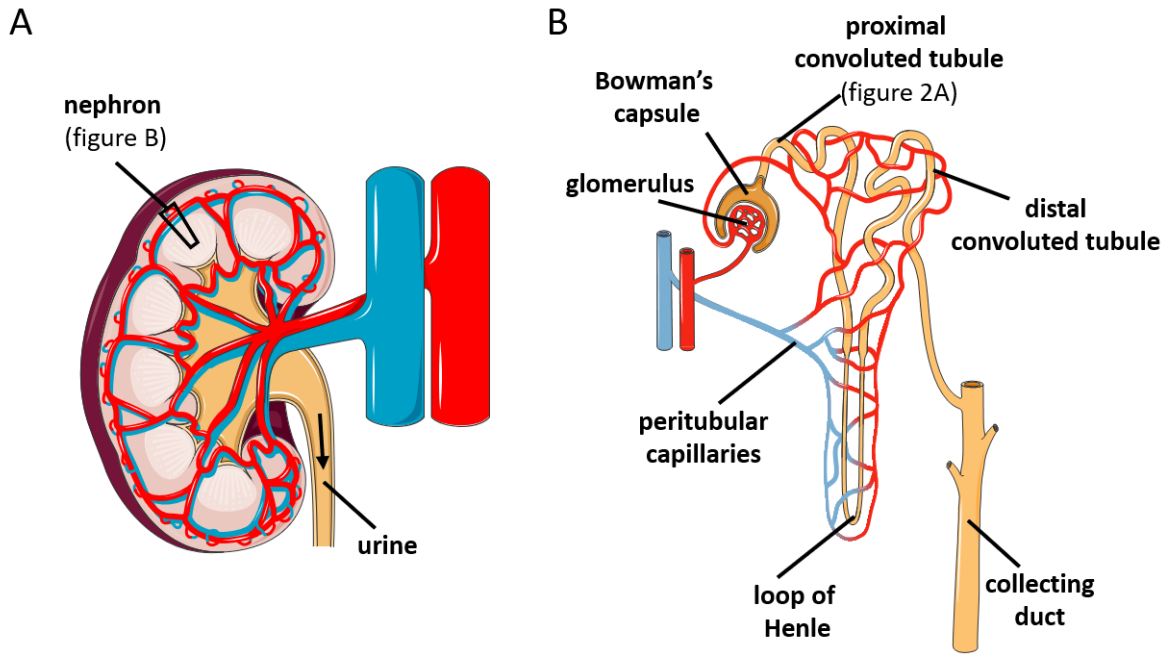

Figure 1: Anatomy of the Kidney, nephron, and transport function of the proximal tubule. A *Sagittal Cross section of one kidney.* ***B*** *Schematic of an individual nephron. The proximal tubule is positioned directly behind the glomerulus. Both are located in the cortex, while the rest of the nephron is primarily located in the medulla. Images adapted from* [28] *and produced using templates provided by Servier Medical Art* [29].

The basal side of the proximal tubule attaches to the ECM via its basement membrane and the apical side of the cells faces the tubular lumen. In the proximal tubules approximately 75% of salts and water and up to 100% of organic solutes, such as glucose and amino acids, are reabsorbed. To be able to facilitate this reabsorbing function the luminal surface is covered by a brush boarder packed with microvilli, enlarging the surface in the range of 20-fold [31]. Each cell is endowed with a single primary cilium, which has a sensory function, recognizing flow or mechanical stimulation [32], [33] (fig. 2 A). The proximal tubule cells are connected to each other by tight junctions which facilitate the barrier function of the proximal tubule layer [34].

The transport of most solutes is facilitated by transport proteins which are located on both the apical and basal side of the proximal tubules [23]. These transporters are classified into two main families: transporters of the solute carrier (SLC) family which use an electrochemical gradient and transporters of the ATP binding cassette (ABC) family which are functioning by hydrolyzing ATP to receive the energy needed [28], [37]. SLC transporters are capable of importing or exporting substrates depending on concentration gradients, while most ABC transporters are mainly responsible for the efflux.

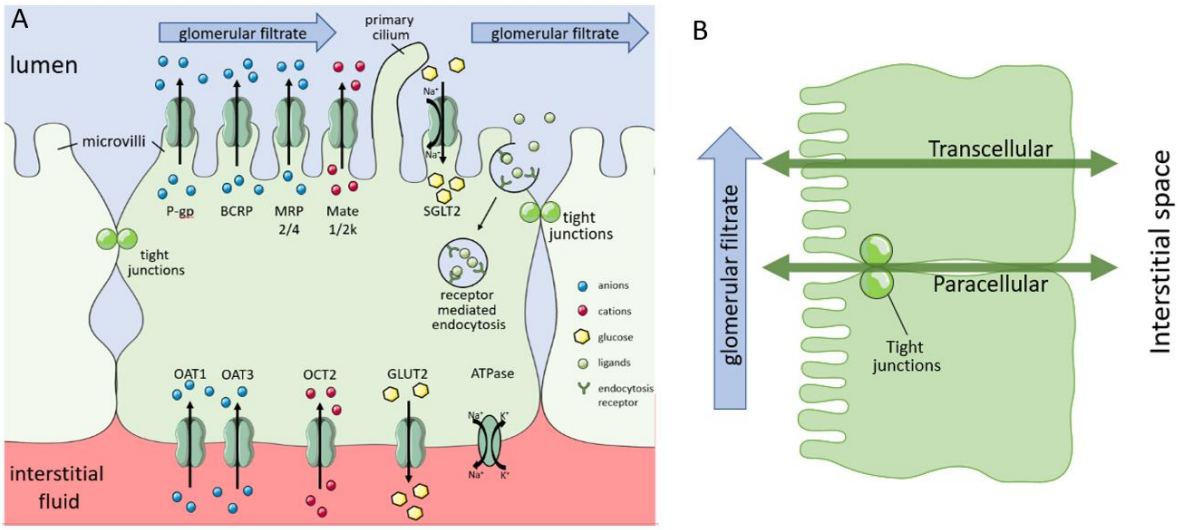

***Figure 2**: **Mechanisms of compound and fluid and transport between the proximal tubular lumen with the glomerular filtrate and the interstitial space. A** Via active processes, cells secrete anions (blue) and cations (red) from the interstitial fluid into the cells and emit them out of the cells on the apical side of the cells into the glomerular filtrate. Glucose (yellow) is reabsorbed from the glomerular filtrate and released back into the interstitial fluid. Ligands (green) are reabsorbed by receptor mediated endocytosis. Adapted from* [28], [35]. ***B** Transport mechanisms of the proximal tubule on cellular level showing the difference of transcellular transport and paracellular transport. Adapted from* [36]. *Figures are produced using templates provided by Servier Medical Art* [29].

Reabsorption function of the proximal tubule

Glucose is the main energy supply of the human body. During the filtration of the blood via the glomerulus, glucose is not removed and therefore available in the glomerular filtrate in the same concentration as in the blood. However, almost all the glucose in the filtrate is reabsorbed by the proximal tubules [38]. The proximal tubules therefore play a crucial role regulating the glucose levels in the blood plasma. 90 % of glucose is taken up from the glomerular filtrate into the cell cytoplasm via SLC transporters sodium-glucose transport protein (SGLT/ SLC5A) 2 and 10 % via SGLT1 [38]. Studies using SGLT2 knock out mice show that even though SGLT2 plays a predominant role in the uptake of glucose [39], [40] it seems that sodium-glucose transport protein 1 (SGLT1) can serve as a partial substitute for SGLT2 [40]. Transport via SGLTs has to follow a sodium (Na+) gradient while Na+ and glucose are co-transported across the apical cell membrane [35]. The Na+ concentration gradient is restored via the sodium–potassium pump (Na+/K+-ATPase) which maintains the Na+ and K+ gradient across the cell membrane while hydrolyzing adenosine triphosphate (ATP) [41]. Transport across the basolateral membrane into the interstitial space and the blood stream is facilitated by diffusion glucose transporter (GLUT/ SLC**2**A**2**) 2 [35] (fig. 2A).

Similar to the reabsorption pathway of glucose, amino acids, phosphate, citrate, and lactate are transported by also using Na+ co-transporters of the SLC family to enter the proximal tubule cells

and diffusion via passive transporters back into the bloodstream [42].Re-uptake of nutrients, proteins such as carrier proteins like albumin, and other small bioactive molecules is facilitated via receptor-mediated endocytosis through multi-ligand receptors cubilin and megalin, which are expressed on the apical side of the proximal tubules [28], [43] (fig. 2A).

Reabsorption of water is facilitated via auquaporin-1, the most important water-transporting protein in cell membranes of the kidney proximal tubule [44](fig. 2A). The extensive water reabsorption leads to a high concentration of chloride (Cl) ions in the filtrate. Reabsorption is facilitated by Cl-formate exchangers into the cells followed by diffusion back into the blood stream, before the filtrate leaves the proximal tubule [44].

Renal clearance function

Next to the reabsorption function of the proximal tubules, substances are cleared from the body into the urine. These include waste metabolites, endogenous and exogenous toxins, such as drugs. The epithelial cell membrane of the proximal tubules functions as a selective barrier between the interstitial fluid on the basal side of the cells and luminal fluid on the apical side. The epithelial cells are connected to each other by tight junctions (fig. 2 A). There are two main transport pathways: paracellular transport and transcellular transport (fig. 2 B). As the proximal tubules are not completely leak tight [31], [34], fluids and solutes are not only transported via the transcellular transport, but also paracellular [36] (fig. 2 B). Transcellular transport is mediated across both the apical and basal membrane through the cellular cytoplasm. Most drugs and waste metabolites are eliminated from the body into the pre-urine cells via transcellular transport. Influx via the basal membrane into the cell cytoplasm and efflux into the glomerular filtrate is mediated by different transport mechanisms. Downregulation or variations of the transport function of one of these transport pathways can lead to either drug accumulation in the plasma or in the proximal tubules itself, which makes the proximal tubule cells a target for drug-induced toxicity [30], [37], [45], [46].

[28], [37]The most abundant influx transporters located on the basolateral membrane of the proximal tubules are organic anion transporter 1 (OAT1/SLC22A6), organic anion transporter 3 (OAT3/SLC22A8), and organic cation transporter 2 (OCT2/SLC22A1) (fig. 2 A). Apical secretion into the lumen is facilitated via the P-glycoprotein 1 (P-gp/ABCB1), breast cancer resistance protein (BCRP/ABCG2), multidrug resistance-associated protein 2 (MRP 2/ABCC2) and 4 (MRP 4/ABCC4), and multidrug and toxin extrusion protein 1 (MATE1/SLC47A1) and 2-k (Mate 2-k/SLC47A2) [28]. Influx of organic cation into the cells is generally facilitated via OCT2 and excreted via MATE1 and MATE2k [28]. An example of such an organic cation is the well-known nephrotoxicant cisplatin. The transport function for cisplatin of the MATEs is less potent then the transport function of OCT2, resulting in an accumulation of cisplatin inside of the cells causing nephrotoxicity [23], [46]. Organic anions are typically transported into the cells by OAT1 and OAT3 followed by a release

into the glomerular filtrate via P-gp, BCRP, or MRP2/4. An example of a drug which is eliminated from the body via OAT1 and MRP4 is the antiviral drug tenofovir [47].

The characterization of a functional proximal tubule model

Before being able to perform advanced experiments on *in vitro* systems it is of high importance that the cultures are phenotypically characterized. Throughout this thesis all developed models were analyzed for the characteristics mentioned in the previous chapter using immunofluorescent (IF) stainings. IF utilizes fluorescent-labeled antibodies in order to identify specific target proteins (antigens) in biological tissue [48]. These antibodies can be then visualized using a fluorescent microscope. For the visualization and correct localization of the primary cilia, stainings were performed using an antibody against acetylated tubulin [49]. To detect the microvilli of the epithelial cells an antibody against ezrin was used, as this protein is concentrated in the microvilli [50]. Zonula occludens (ZO)-1, also known as the tight junction protein [51], was used to visualize the barrier formation of the tubules. Next to a visualization of the tight junctions their functionality can be tested using assays which assess the barrier function of the cell layer. A tight barrier formation of proximal tubule layers is important to be able to monitor the effect of compounds on the barrier formation, but it is also crucial when the transport of compounds across the cell layer is assessed. The barrier formations can for example be monitored by studying a fluorescent labeled compound (e.g. a Fluorescein isothiocyanate (FITC) dextran) under the microscope, which was added to the lumen of the tubule. If the dye does not leak through the cell layer of the tubule, the cell barrier can be considered to be leak tight. A good barrier integrity and correct polarization of the tubule is essential for assessing transport and directional toxicity as it allows interrogation and exposure of the apical and basolateral sides independently from one another [31]. Another possibility to assess the barrier function of the proximal tubule layers is by measuring the TransEpithelial/Endothelial Electrical Resistance (TEER). Here, the tightness of the barrier function of the cell layer is assessed by measuring the associated electrical impedance [52]. In addition, assays which can be used to analyze an expected response of the model to certain compound treatments need to be tested or evolved. This can for instance be done using compounds which are known to trigger AKI or inhibit the transport function across the cell barrier. Only when all these examinations are successful, the model can be used for the investigation of a variety of compounds and eventually disease models can be developed.

High-throughput *in vitro* models of the proximal tubule - from 2D to 3D

Up to today the gold standard of *in vitro* models are 2D models, with and without a supporting ECM. These 2D models can be cultured at a large scale in multi-well plates which offer up to 1536 separate identical cell cultures for drug testing. Cells are seeded in these plates and after a few days compound screenings can be performed. This kind of upscaled tests offer important insights in early drug discovery as well as predicting possible drug concentrations in further experiments. However, these systems lack the complexity of the human body as cell monolayers are attached

to (coated) surfaces with their basal side attached to the culture dish (fig. 3A). These models clearly lack the third dimension on the basal side of the cells which, among others, receives all reabsorbed water and glucose in the proximal tubules [53]. Moreover, the proximal tubules play an important role in eliminating drugs into the luminal fluid on the apical side. Therefore, it is crucial for these cultures to be grown in a way that they can be accessed from both the basal and the apical sides. In the last years, a more complex *in vitro* system started to dominate the market: the Transwell® system. Cells in these systems are grown on ECM-coated artificial porous membranes, enabling access to the cell layer from the apical as well as from the basal side [54], [55]. An example of such a culture system can be seen in figure 3B. In Transwell systems, up to 96 cultures can be grown in parallel. This is still a high number of replicates per plate, which means that these systems can be used for high-throughput studies. However, models of proximal tubules cultured on these devices lack the renal proximal tubule typical phenotype and behavior (e.g. correct polarization or significant receptor-mediated transport) which is shown to be a deficiency of shear stress the cells need to experience [56]–[58].

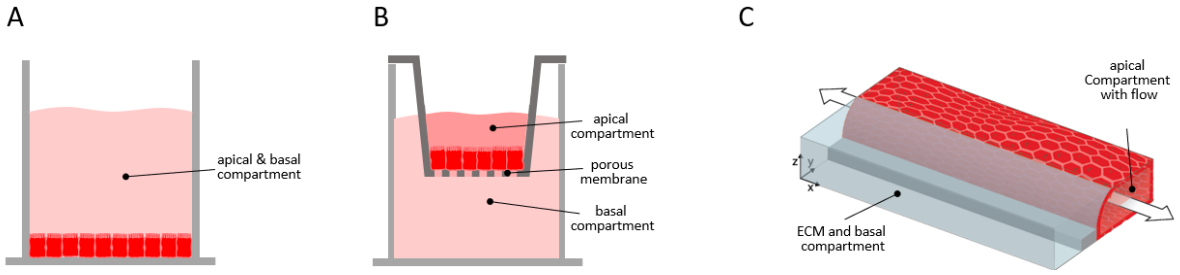

Figure 3: From simple to complex: High-throughput culture systems for proximal tubule epithelial cells based on a standard multiwell plate layout. A *Typical 2D culture system in a standard multiwell plate. A monolayer of epithelial cells is grown on a plastic surface of a culture dish. Medium supply is offered from one side (top) only. Slightly more physiologically-relevant models provide a layer of extracellular matrix (ECM) between the cells and the surface. Multiwell plates can offer up to 1536 separate culture chambers.* ***B*** *Transwell® system with up to 96 culture chambers. In a Transwell system, cells are cultured in an insert on top of an ECM-coated porous membrane. This way of culturing enables access to the basal as well as the apical side of the cell layer.* ***C*** *OrganoPlate® 2-lane system containing 96 culture chambers. The OrganoPlate is a newly developed system which enables the possibility to culture epithelial cells against an ECM in a perfused, membrane- free 3D setting.*

In 2013 the OrganoPlate® 2-lane (Figure 3C) was launched which enables parallel cultures of up to 96 tissues to be grown in a membrane-free 3D environment with perfusion flow. This system was a breakthrough for culturing endothelial vessels membrane free in a high-throughput fashion against a collagen 1 gel [59]. Though the cells do grow layers against a freestanding ECM, this system was not suitable for culturing proximal tubules, as it lacks the possibility to access the cell layer growing against the ECM from the basal side of the cells. This access is particularly important for the proximal tubules as they actively transport compounds via the cell membrane.

Using microfluidic high-throughput systems for the culture of proximal tubules

In the last decade, a variety of 3D cultured models of proximal tubules has been developed. The majority of these models is cultured in microfluidic channel structures on chip systems. These microfluidic channels can be fabricated using different approaches. Jang et al. [57] for instance, published a model of a kidney-on-a-chip device with cultured renal proximal tubule cells (RPTEC) on a perfused porous polyester membrane microfabricated on a polydimethylsiloxane (PDMS) chip. Fluid sampling can be performed from the apical chamber containing the RPTECs and the adjacent basal chamber. Jang et al. compared their model directly with the Transwell system and could conclude from the results that the presence of flow is crucial for a wide range of physiological functions. Using a model of perfused hollow fibers made of ECM-coated polyethersulfone, Jansen et al. [60] could demonstrate active transport via the OCT2 transporter across the membrane. Homan et al. [56], as well as Weber et al. [58] both used a system where cells from the proximal tubules were successfully grown against an interface of an ECM gel. What all these systems have in common is that the lumen of the proximal tubules could be perfused with exposure to shear stress, the cells were constantly supplied with nutrients and oxygen and waste products were removed. All these models offer access to the basolateral side of the cell membrane, which is of high importance for transport studies across the membrane or independent exposures of the apical or basal cell membrane.

As was discussed in the previous subchapter, one important aspect which all these models lack is the possibility to use these microfluidic systems in a high-throughput fashion similar to the OrganoPlate 2-lane. The patterning of the channel structure on the OrganoPlate is not fixed but can be modified, as long as the design allows access to the channels via the 384 top-well plates. This offers the opportunity to develop different models. One of these models, which offered the high throughput possibilities of the OrganoPlate 2-lane in combination with the possibility of growing tubules with access to the apical and basal side of the cell barrier was the OrganoPlate® 3-lane system. The OrganoPlate 3-lane was developed and continuously improved during the research of this thesis. This development can be also reviewed in more detail in the doctoral thesis by S.J. Trietsch [61].

In short: the OrganoPlate 3-lane (figure 4A) comprises 40 chips (figure 4B) in one platform with a microfluidic channel system that is embedded between two microscope-grade glass plates. Access to this system is enabled via the wells of the top plate. These wells are also functioning as reservoirs, which provide fresh medium and waste product dilution for the cultures in the microfluidic system. For establishing 3D cultures in the chips, a liquefied ECM is added to the middle channel (fig. 4 C). By microfluidic forces, the ECM is guided into the system and patterned via meniscus pinning between the two phaseguides. After polymerization of the ECM, cells are seeded to one of the adjacent channels and medium is added to all wells which are connected to

the perfusion channels. By placing the plate on a rocker platform, perfusion flow through the system is started by passive leveling of the medium (figure 4D).

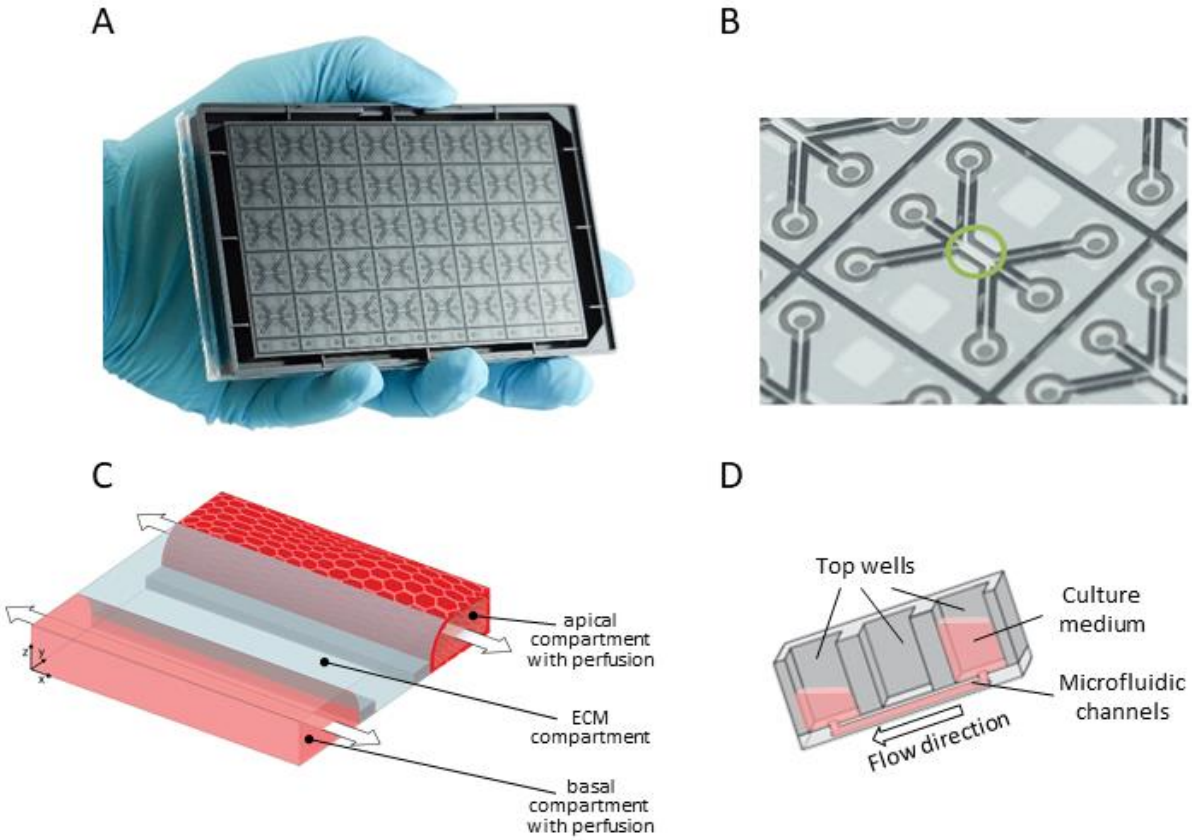

Figure 4: The OrganoPlate® 3-lane. A *Bottom view on the modified bottom of the OrganoPlate 3-lane system containing 40 microfluidic cell culture chips embedded in between two microscope grade glass plates. Each single chip can be accessed from the top via the microtiter plate.* ***B*** *Zoom in on one 3-lane chip comprising the three channels in the center (green circle).* ***C*** *Artist impression of the center of the OrganoPlate 3-lane. Channels are divided by small ridges called PhaseGuide (grey). After loading a gel (light blue) in the middle channel, cells (red) are seeded to one of the adjacent channels. After cell attachment, medium is perfused (indicated by white arrows) in both the lumen of the tube and the second perfusion channel (light red).* ***D*** *Side cut view of the top wells connected by one of the microfluidic channels. Perfusion of cell culture medium through the system is created by placing the plate in an angle position on a rocker platform. This results in positioning the wells of the plate on different heights enabling a gravity-driven flow though the channels.*

Triggered by the perfusion, monolayers of the epithelial cells grow against the interface with the ECM and the walls of the microfluidic channels until a confluent tubule is grown. These tubules can be used for further experimental studies, including the assessment of the cell barrier against the ECM.

In this thesis the use of the OrganoPlate 3-lane system for the culture of the proximal tubules is described. The 3-lane system offers the possibility to grow proximal tubule cells against an ECM gel. To mimic the tubulointerstitium of the kidney collagen type I, which is one of the most

abundant proteins in the tubulointerstitium of the kidney [62], [63], was used. Cells were seeded into the top channel of the OrganoPlate 3-lane and under bidirectional flow through the perfusion channels confluent tubules were grown. Assays were performed from day 6 to day 10.

Scaling up of the model complexity to a higher physiological relevance: Cocultures of the proximal tubule

The proximal tubules are only one part of the nephron; they are surrounded by a panel of other cell types which also play a role in its function. They interact most closely with endothelial vessels (peritubular capillaries, fig. 1B) which directly provide all compounds that need to be eliminated into the urine via the proximal tubules.

There is a growing number of research papers that describe the importance of adding endothelial cells to the proximal tubule monoculture models. The first papers which demonstrated the benefits of culturing cells in cocultures were already published before 2000. Linas and Repine for example could showed in 1999 [64] that the function of the proximal tubules is controlled by endothelial cells. Aydin et al. demonstrated in 2007 [65] that the human microvascular endothelial cell line HMEC-1 influences the behavior of the renal epithelial cell line HK-2 when grown in a coculture separated by a filter membrane. They measured a significantly higher barrier function of the epithelial cells. Tasnim and Zink in 2011 [66] published that essential transporters of the proximal tubules got upregulated in primary renal proximal tubular cells when cocultured with endothelial cells. Anion transporter OAT 1 showed relative expression levels of a fold change of around 5 when cells were cultured in a coculture compared to the monoculture.

In the studies mentioned above endothelial cells and epithelial cells were cultured in the same system. Mainly Transwell-like systems (fig. 3 B) were used with one cell type cultured on the surface of the bottom compartment and the second cell type on the filter membrane of the top compartment. These systems lacked at least one of two physiological relevant aspects: perfusion flow on the apical sides of both membranes and the correct physical constellation with the basal membranes of each of the structures facing each other.

A handful of systems, which combine both requirements for culturing renal epithelial tubules in a coculture set up with endothelial tubules have been published in recent years (fig. 5).

Vedula et al. [67] developed a device which offers the possibility to culture two different cell types in chambers separated by a polycarbonate membrane under perfusion flow (fig. 5 A). In their device, interaction between both cell types is possible as the membrane is permeable for liquids and solutes, which enables the reabsorption and the transport function across both cell barriers. The membrane itself is topographically-patterned to facilitate tissue organization and function, which they demonstrated by performing a glucose reabsorption study. Rayner et al. [68] (fig. 5 B)

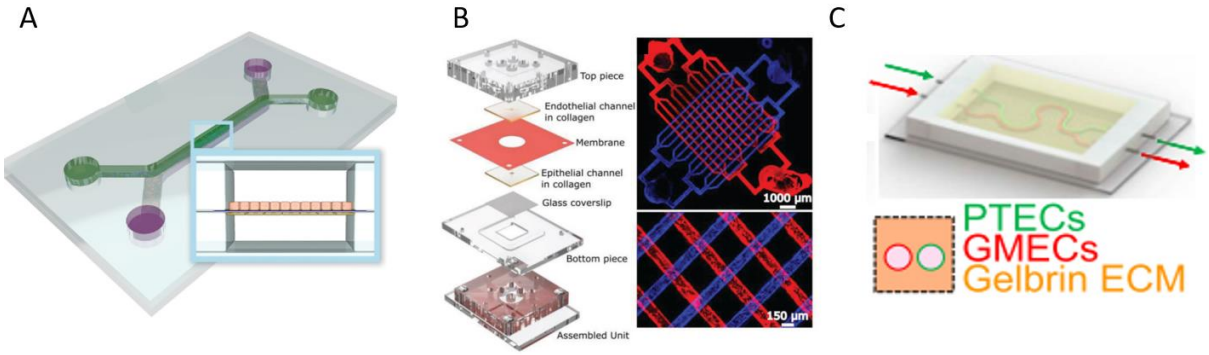

Figure 5: Microfluidic devices developed for culturing perfused cocultures of renal epithelial tubules and endothelial cells. A *Vedula et al. developed a device where the two perfused channels (green for RPTEC and purple for the endothelial cells) are separated by a polycarbonate topographically-patterned membrane with pore structure.* [67] ***B*** *Parallel channel networks of two separately perfused units embedded and separated by a collagen gel only. In one of the two channel networks renal cortical cells were seeded, the other network was utilized for endothelial cells* [68]. ***C*** *Proximal tubules (PTECs, green) and endothelial cells (GMECs, red) grown in close proximity in a bioprinted ECM network with no artificial barriers present* [69].

and Lin et al. [69] (fig. 5 C) used a similar approach to culture both epithelial and endothelial cells in close proximity by patterning the tubules and vessels inside of an ECM network. Rayner et al. used collagen whereas Lin et al. used a mixture of gelatin and fibrinogen. Both research groups could show that the ECMs were permeable for albumin and glucose by performing successful reabsorption studies. However, so far none of the introduced devices for cocultures can be used in a high throughput fashion which would enable the possibilities to examine the toxicity of broad concentration ranges and compound libraries. Remarkably, Rayner et al. could show that their device is not only suitable for culturing cocultures, but they also already performed triple culture experiments by incorporating pericytes embedded into the ECM [68]. This approach is already a first interesting step in the direction of a complete nephron on a chip as the interstitial cells such as fibroblasts, pericytes, or immune cells are expected to support the regeneration and function of the renal epithelium [70].

Aim and outline of the thesis

Current pre-clinical studies assessing nephrotoxicity lack human-derived *in vitro* models which accurately predict the response of the *in vivo* situation. The aim of the research described in this thesis was to develop a physiologically relevant 3D *in vitro* model of the human renal proximal tubule which combines its physiological complexity with a robust high-throughput organ-on-a-chip system. This proximal-tubule-on-a-chip model should be able to mimic the *in vivo* situation for a variety of applications, such as drug assessment in nephrotoxicity studies, drug-drug interaction studies including drug transport studies, and the assessment of compounds which protect against tubular damage. This thesis focused on the establishment and characterization of such a model and relevant assays. The ultimate aim was to offer a tool for drug research and development of novel medications that is accessible to academia and industry.

In **chapter 2** our aim was to develop a 3D perfused human proximal tubule model that can be used for nephrotoxicity and renal transport assessment. We investigated whether renal proximal tubule cells (RPTEC) can be cultured as tubular structures with correct polarization in the OrganoPlate 3-lane. We elaborated on the need for shear stress, and how fluid flow rate and induced shear stress in the microfluidic channels of the OrganoPlate can be calculated. Furthermore we examined if the model can be used to evaluate the nephrotoxic effect of cisplatin, a drug which is known to show a damaging effect on the proximal tubules *in vivo* as well as *in vitro*. Moreover, our aim of this chapter was to show that the model can be used to study the transport function of the proximal tubule. We demonstrated the transport function using two different approaches: transport of compounds which are taken up into the cells, as well as transepithelial transport assessment across the membrane. In this chapter, a model of the proximal tubule was developed which can be used for drug screening studies and can serve as a solid foundation for further model development of the proximal tubules.

The development of the proximal tubule model described in **chapter 2** was part of the Nephrotube challenge crackIT which was organized by the National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3Rs) [7] and sponsored by GSK, Pfizer, and Roche. The aim of the challenge was to develop a high-throughput, 3D microfluidic platform (Nephroscreen) for the detection of drug-induced nephrotoxicity [71]. In parallel with the work described in **chapter 2**, Vriend et al. [72] developed a screening platform for drug transporter interaction using their conditionally immortalized proximal tubule epithelial cells overexpressing organic anion transporter 1 (ciPTEC-OAT1) seeded in the OrganoPlate. Suter-Dick et al. [73] developed a method which combined the determination of miRNA and the usage of the OrganoPlate system. The aim of **chapter 3** was to develop an advanced proximal tubule on a chip model combining different cell types and readout assays. This was achieved by a joint study of all three research lines within the Nephroscreen to integrate all assays in one platform to study nephrotoxicity induced by known and unknown drugs. Tubules grown in the OrganoPlate were exposed to four model compounds and a panel of eight unknown compounds provided by the sponsor consortium. Proximal tubules grown in the 3-lane system can be used for a complex drug screening combining different cell types with a huge panel of different assays.

In chapter 4 the aim was to investigate whether *in vivo* observations can be replicated with our model developed in **chapter 2** and **chapter 3**. For the study, the effects of the two HIV-medications Stribild and Genvoya on the proximal tubules of the kidney were analyzed. Both drugs contain two different pro-drugs of the known nephrotoxicant tenofovir (Stribild, tenofovir disoproxil (TDF) and Genvoya, tenofovir alafenamide (TAF)) in combination with the same concentrations of Elvitegravir, Cobicistat, and Emtricitabine. In the recent years Stribild was replaced by Genvoya as a result of a better side effect profile. Toxicity of the tenofovir variant used in Genvoya was picked up at only physiologically irrelevant concentrations suggesting that

our model is able to pick up the reduced nephrotoxic effect of TAF compared to TDF. Next, we investigated whether our model is suitable to pick up cumulative toxicity when combining the tenofovir variants with either Elvitegravir, Cobicistat, or Emtricitabine. Additive to super-additive synergistic toxicity could be clearly shown for some combinations, whereas single dosages of each of the compounds showed a lower toxicity.

After using a mono-culture model of proximal tubule cells in **chapters 2** to **4** our aim in **chapter 5** was to explore whether an endothelial vessel alongside the proximal tubule can be added to investigate if AKI induced by ischemia can be studied on this co-culture model. The use of the coculture as an acute kidney injury (AKI) model was characterized by an immunofluorescence staining and validated by a nephrotoxicant study. Subsequently, the ischemic AKI model was developed by exposing the coculture to different ischemic conditions. Two ischemic conditions (condition 1: glucose-free basal medium, static, low oxygen and condition 2: glucose-free basal medium, perfusion, low oxygen) were selected. A co-incubation with potential protective compounds succeeded to show a significant protection against tubular damage after addition of adenosine to the culture medium. This study led to a functional coculture of epithelial tubules and endothelial vessels which can be used to study renoprotective compounds on an ischemic induced AKI model.

In **chapter 6** a general conclusion was given, including a summary of each of the chapters. Finally, this was followed by an outlook on how to address future research on the culture of proximal tubule *in vitro* models.

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