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

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Chapter 6

Overall Discussion, Future Perspectives, and Summary

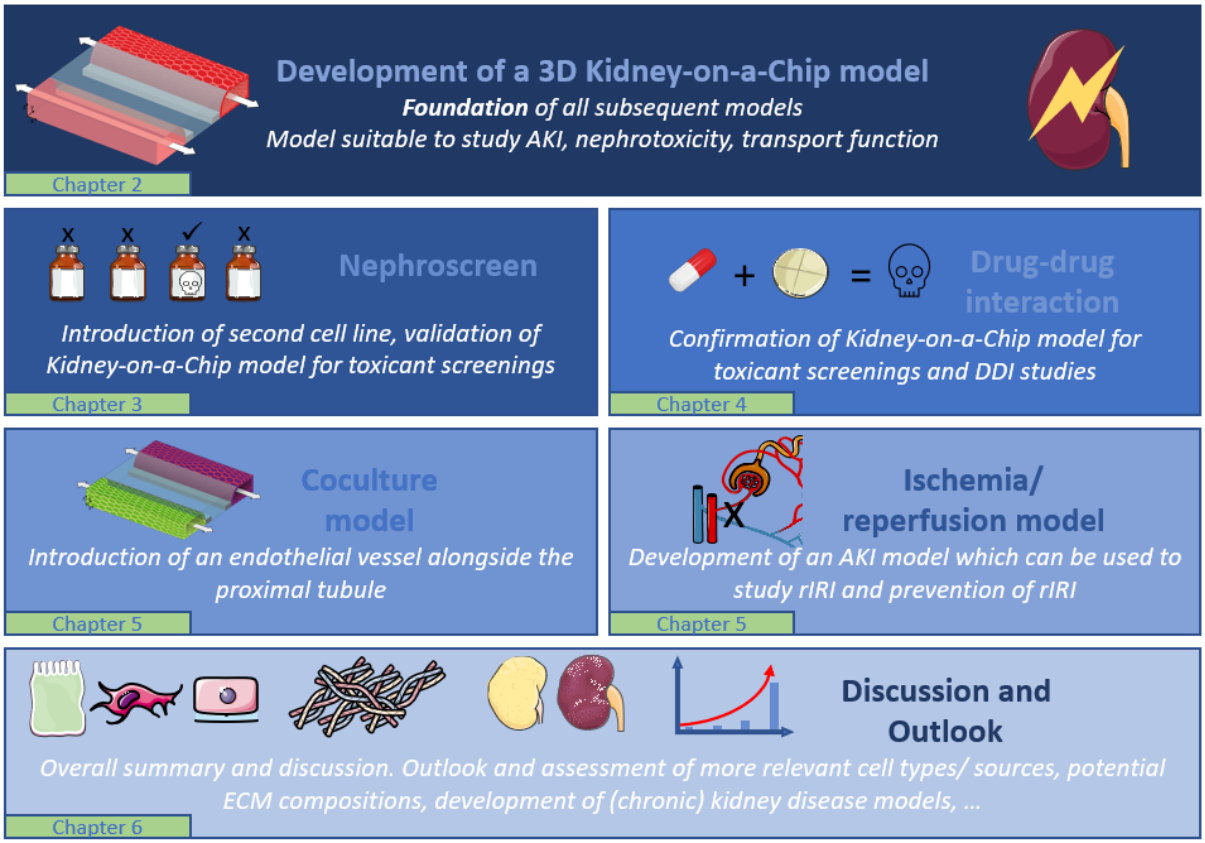

Figure 1: Development of the Kidney-on-a-Chip, the initial model (chapter 2), which was a general foundation for all subsequent models developed over the course of this thesis. The initial model was validated as a screening platform through a panel of 12 different compounds on 2 proximal tubule cell sources (chapter 3). To be able to broaden the sensitivity of the system a second proximal tubule cell source, ciPTEC-OAT1 was introduced. Using knowledge obtained in the toxicant study a Drug-drug-interaction (DDI) study was performed which confirmed the stability of the model for testing nephrotoxic drugs (chapter 4) and their interaction. In Chapter 5 a coculture model was introduced by adding an endothelial vessel adjacent to the proximal tubule. This model was subsequently used to study renal ischemia reperfusion injury of the tubule and the vessel. In chapter 6 a summary of the thesis including a general conclusion is provided. Suggestions for new research areas are discussed which can be undertaken using the model developed here.

Development of a kidney-on-a-chip platform

The number of 3D human tissue models increased remarkably during the last years. This has been a development driven by the limitations of 2D cell culture, the strive to reduce and replace animal testing, and the small percentage of new drug compounds which enter clinical trials. 2D cell culture models of the kidney have not accurately predicted nephrotoxicity, as cells lack some physiologically-relevant characteristics. These characteristics include correct polarization and the possibility to culture on a permeable surface which resembles the extracellular matrix (ECM) with its interstitial fluid on the basal side and the exposure of the cells to shear stress on the apical side of the cell membrane [1], [2]. There are both ethical and physiological reasons to reduce animal testing in drug research. Pressure from society, but also rising costs of animal studies, support the aim to develop alternatives [3]. In addition to that, there are significant differences between physiology of humans and animals, as example for the kidney there is a difference in expression of important drug transporters [4], [5]. Finally, when animal models incorrectly predict human response to the tested drugs and toxicity is only detected subsequently in clinical testing this can lead to high costs or even worse to health-threatening problems in humans [6].

The aim of this thesis was to develop an *in vitro* model with which it is possible to study safety and efficacy of a range of drugs. We hypothesize that such an *in vitro* model at the very least has to offer the following key aspects which we consider crucial for a successful nephrotoxicity-predicting test platform:

- 3D culture of epithelial cells
- Fluid shear stress
- Correct polarization of cells
- Leak tight barrier formation against a natural surface
- No artificial membranes
- Apical and basal access
- High-throughput platform
- Renal cell source expressing key transporters of proximal tubule cells
- Coculture possibilities
- High reproducibility of experimental outcomes
- Commercial availability

The aim was to characterize the model by testing the safety and efficiency of known drugs. In **chapter 2** we describe the development of a model which combines most of the listed points. Human renal proximal tubule cells (RPTEC) were seeded to form tubular structures against a collagen 1 gel into the OrganoPlate® 3-lane. Fluid shear stress was resembled by introducing a gravity-driven flow through the system after placing the OrganoPlate on top of an interval rocker system. In the supplementary data set of **chapter 2** we showed that perfusion is crucial for tube

formation. However, the importance of flow is not only important for the tube formation, but it was also shown that fluid shear stress increased the transport capability of proximal tubule cells grown in the OrganoPlate [7]. Similar observations were published by several other authors, showing that shear stress indeed affects the phenotype and transport function of proximal tubule cells [8]–[13].

For the development of our model, we used a commercially available kidney RPTEC control cell line (SA7K clone). We could demonstrate that these cells exhibit correct polarization and formed leak tight barriers. Polarization of cells was confirmed by immunofluorescence staining depicting one primary cilium per cell pointing to the lumen of the tubules. Cilia function as sensors for fluid flow and as a controlling unit for cell proliferation [14]. Moreover, microvilli, which are covering the brush boarder of the epithelial cells, were exclusively present on the apical surface. Microvilli are responsible for mechanosensing the flow [15] and they play an important role increasing the apical surface for better re-absorption capabilities [16].

Using an immunofluorescent staining and a barrier integrity assay, it was demonstrated that the RPTEC formed leak tight barriers. Tight junctions were found at cell-cell connections, which were visualized using the tight junction marker zonula occludens (ZO)-1 [17]. The tightness of these junctions was validated by a barrier integrity assay which we developed [18], [19] in the course of this thesis for the OrganoPlate system. The tubules were flushed with a medium which contained fluorescent dextran dyes of different sizes. The apparent permeability of the different sizes of dextran could be calculated by measuring the intensity of the dyes at both sides of the epithelial barrier, followed by calculating the ratio of the receiving ECM compartment behind the barrier and the perfused channel. The barrier integrity assay opened a vast array of possibilities which we could use to analyze our model. We used it to determine at which time point after seeding the tubules grew tight barriers against the collagen 1 gel and exposures could start. Furthermore, we could use the barrier integrity assay for time-to-leak experiments by monitoring real-time when the barrier function was affected by a toxic compound [20]. For the analysis of the proximal tubules, we mainly used the assay as an endpoint assay after a toxicant exposure. Just recently, a new device to measure the tightness of barriers was developed for use in the OrganoPlate: A machine which is able to measure trans-epithelial/endothelial electrical resistance (TEER) of the cell barriers formed against the collagen 1, called the OrganoTEER® [21]. The OrganoTEER can already be successfully used to study the differences in tightness of cell barriers with high TEER values. Proximal tubules are known to be a comparably leaky epithelium with very low TEER values of 6-10 ohm/cm^2 [22]. In the first experiments we could already measure values which fall into this range, though we could not use the device for analyzing the barrier function after toxicant exposures, as these values are around the detection limit of the analyzing software. However, further work which will ensure that the OrganoTEER will be able to pick up differences

in barrier tightness of even small TEER values in the near future is ongoing, and promising results to this end are already achieved.

The proximal tubule-on-a-chip model was set up in the OrganoPlate 3-lane, which enables access to the tubule from the basal and the apical side. Combining this important specification with the characteristics of correct polarization and leak tight barriers as described above, the platform can be used as an ideal model for trans-epithelial transport experiments. Two different methods were used to show active transport of cationic compounds : measuring the compound concentration intra-cellular and trans-epithelial transport across the cell membrane. The transport function could be successfully inhibited, proving the existence of active transport. Even though the transport function of cations by the RPTEC (SA7K clone) could be shown, these cells lack some of the key transporters which are crucial for transporting anions. In **chapter 3** a second epithelial cell source was introduced to overcome this problem: conditionally immortalized proximal tubule epithelial cells overexpressing xenobiotic organic anion transporter 1 (ciPTEC-OAT1) [23]. Using ciPTEC-OAT1 we were able to set up a test platform which we could use to detect the toxicity of both active transport dependent drug types; cations and anions. By combining the two cell types with a broader panel of compounds and readout assays a screening platform was developed, called the nephroscreen. We could show that the platform and the protocols are robust and reproducible. In parallel with the development of the nephroscreen a drug-drug interaction (DDI) study was performed on RPTEC, as described in **chapter 4**. The study in **chapter 4** was designed to determine if we can use our proximal tubule-on-a-chip model in a DDI study using a panel of multiplexed assays to examine the toxicity of single and combined dosages of human immunodeficiency virus (HIV)-targeting drugs. HIV-patients are commonly treated with the drug Genvoya which replaced Stribild in the recent years. Both are tenofovir-containing medications and are supplied as pills with a combination of active compounds, namely emtricitabine, elvitegravir and cobicistat, and one of the prodrugs of tenofovir, tenofovir disoproxil fumarate (TDF) or tenofovir alafenamide (TAF). Of all the ingredients in these pharmaceutical formulations, in particular the drug-related nephrotoxicity of tenofovir is widely accepted. Our goal was to investigate if we are able to detect a toxic effect on the proximal tubule cells by one of the two tenofovir prodrugs, especially when combined with one of the additive compounds. One major difference between these two prodrugs is that TDF is not stable when in contact with human plasma, where it quickly undergoes hydrolysis into parent tenofovir, whereas TAF has a much higher stability which results in a higher antiviral activity and reduced dosages compared to TDF. In **chapter 3** we could show that the parent form of tenofovir had a cytotoxic effect on ciPTEC-OAT1 whereas no effect could be measured on RPTEC. As the parent form of tenofovir is transported via the anion pathway (mainly via OAT1) into the cells this was an expected result. However, in the present study we used RPTEC. When we tested the two prodrugs, TDF did not lead to a damaging effect on the proximal tubules, which was expected considering that TDF is hydrolyzed into the parent form of tenofovir when in contact with culture medium. Interestingly,

TAF showed a damaging effect to the proximal tubules at similar concentrations which we found to be toxic for parent tenofovir in the OAT1 expressing cells. These results showed us, that the compound TAF is indeed stable when in contact with serum and is not hydrolyzed into the parent form of tenofovir. Moreover, it is very likely that TAF is not assimilated by the cells via OAT1 transport, which was already suggested before by Bam et al. [24]. Inside of the cells TAF is then converted to parent tenofovir via hydrolysis by intracellular proteases [24], [25].

The two prodrugs of tenofovir are not administered on their own, but in combination with three other antiretroviral agents, namely elvitegravir, cobicistat, and emtricitabine [26], [27]. Next to testing the two prodrugs in single dosages we performed a co-treatment of the prodrugs with the goal to assess if any of them amplifies the cytotoxic effect of the two tenofovir prodrugs. Our results suggest, that even at high concentrations emtricitabine did not have a damaging effect on the proximal tubules, in isolation or in combination with one of the two prodrugs of tenofovir. Elvitegravir did not have an enhancing effect on the cytotoxicity when dosed in combination with TDF. However, when dosed together with TAF elvitegravir strongly enhanced its damaging effect, and super-additive synergistic effects were measured. This result was surprising as elvitegravir is thought to not affect the kidneys but is mainly eliminated via hepatobiliary excretion [28]. To develop a full picture of the toxicity of the co-administration of tenofovir and cobicistat additional research could be undertaken by combining the proximal tubule model with a liver model. When cobicistat was dosed alone, a damaging effect could be already be detected. A combination with TDF led to an increased damage which could be picked up in all assays. A combination of cobicistat and TAF led to an increased cytotoxicity which was detected for the low as well as the high dosed combinations.

The study in chapter 4 indicates that in future studies drugs should be tested in the cocktail they will be administered clinically. However, during *in vitro* experiments some of the drugs might be incorrectly dosed, as *in vivo* they would be metabolized quickly by the liver or cleared via the gut. To overcome this problem a possible solution could be to connect the present model with a liver-on-the-chip system and/or a gut-on-a-chip system. Or, include pharmacokinetic modeling to choose proper concentrations for experiments.

In **chapters 2-4** we mainly focused on acute kidney injury (AKI) as a result of an acute reaction to a nephrotoxicant. Next to these toxicant models we were also interested if we are able to develop a disease model for treatment related toxicity which we can use to screen the protective effect of drugs. To achieve this, we added 3 new aspects to our model which we described in **chapter 5**: coculture with an endothelial vessel, induction of AKI caused by an ischemic insult, and the assessment of compounds that have a protective effect during the ischemic event leading to AKI. For the development of the coculture model, we expanded the kidney on a chip developed in **chapter 2** by introducing endothelial cells to the second perfusion channel. We characterized the culture using immunostaining for cell type specific markers and ensured correct polarization. We

validated AKI-associated read-outs through exposure of the coculture to known nephrotoxicants. To study ischemic events in the kidney the coculture was exposed to ischemia through a combination of low oxygen, reduced glucose, and flow interruption conditions. Subsequently, cultures were reperfused under normal conditions to trigger reperfusion damage. Injury was quantified through morphological assessment, caspase-3/7 activation, viability assessment, and lactate dehydrogenase release. Low oxygen, reduced glucose and interrupted flow were detrimental to the tubules in any combination of two, whereas the combination of all three led to the most severe damage. This effect was strongly amplified upon reperfusion. The effect of ischemic conditions on the endothelium was less severe than on the epithelium.

For the assessment of protective compounds adenosine, nicotinamide, or N-acetylcysteine were added to the culture medium of the proximal tubule and the endothelial vessel during the exposure and reperfusion. Adenosine was shown to have a significant protective effect, whereas no such effect was found for nicotinamide, and N-acetylcysteine.

In **chapter 5** we were able to show that AKI induced by Renal ischemia/reperfusion injury (rIRI) can be effectively modelled in this perfused 3D kidney coculture setup. The robustness of the model and assays in combination with the throughput of the platform make it ideal to study the effect of AKI-preventing compounds and enable development of novel therapeutic modalities.

One unique aspect of the system developed in this study is the commercial availability of the platform, which was also one of the conditions of the crack-IT challenge described in **chapter 3**. This opens the possibility that the platform is not only used and improved by our research team, but it can be rapidly improved, similar to open source software, by an unlimited number of research groups.

Towards a functional nephroscreen

The model which we developed in **chapter 2** as part of a NC3R crack-IT challenge was called the nephroscreen. The development of the nephroscreen was a joint project of a consortium consisting of 4 different parties: Mimetas, Fachhochschule Nordwestschweiz, Radboud University Medical Center Nijmegen, and Utrecht University. The goal of the nephroscreen project was to develop a proximal tubule-on-a-chip platform capable of accurately identifying nephrotoxic effects using human cells (**chapter 3**). The nephroscreen was primarily designed to fulfill the requirements of the sponsors, which were exclusively pharmaceutical companies: developing a platform which can be used to reduce the costs and increase the predictability of pre-clinical studies. During the development of the nephroscreen a second proximal tubule cell source was used: ciPTEC-OAT1 developed by one of the consortium partners. ciPTEC-OAT1 showed a significantly higher sensitivity during transport studies while RPTEC were used when the barrier function of the tubules was of interest. By combining these two cells sources, a compound panel of the proximal tubule of in total 12 compounds was screened for its toxicity. Four of these

compounds were known nephrotoxicants which were also used to set up the different assays. Eight compounds were unknown as they were provided by the sponsors of the study. We could show that the majority of the provided compounds did have a nephrotoxic effect on the nephroscreen. The effect of one compound on the nephroscreen could only be detected during the transport studies as this compound did not have an effect in any other of the other assays. A second compound showed an interaction with P-glycoprotein (P-gp) and a mild effect on an increased level of microRNAs (miRNAs), but again no damaging effect on the viability of tubular integrity of the tubules. One of the compounds did not show any cytotoxicity during the initial study, therefore we tested it in a long-term experiment where the exposure was performed for 11 days instead of 48 hours. This study showed that for some compounds a long term study is able to demonstrate toxicity, which was not picked up in the 48 hour experiment. For future experiments using the nephroscreen it would be recommended to divide the experiments performed on the nephroscreen into 2 phases. In phase 1, a 48-hour long initial testing of a broad panel of compounds should take place, followed by a second phase where only compounds are tested which did not show an effect in the initial phase.

To ultimately install the nephroscreen as a screening platform for drug research at pharmaceutical companies, steps have to be taken to ensure the reproducibility of the experiments. This can be done by implementing standard operating procedures which are in line with the guidelines of Good *In vitro* Method Practices, which were published by the Organization for Economic Co-operation and Development in 2018 [29]. If these guidelines are followed, the platform is ready to be used in short term to test compounds prior to testing them *in vivo*. Here, it would be important to carefully compare the results of each study, *in vitro* on the nephroscreen, *in vivo* on animals and ultimately *in vivo* in humans in clinical studies to investigate the predictive potential of the nephroscreen compared to animal studies. We hope that this will ultimately result in omitting animal tests for drug development and replacing them by *in vitro* models.

As mentioned before, the nephroscreen, including the protocols, the platform, and the cells are commercially available. This will help to improve the suitability of the nephroscreen towards a widely accepted platform during pre-clinical studies. Just recently our system was used to set up a glomerulus-on-a-chip model [30] which shows that our system aligns with the current research interests.

Development of an ischemia model which can be used to study the preventive effect of compounds

Renal ischemia/reperfusion injury, together with nephrotoxicity, is one of the major causes of AKI caused by tubular damage after an insult. In **chapter 5** we continued to develop our model so it could be used to study renal ischemia. Ischemia is a sudden restriction of blood supply of an organ. With our model we could show that not only the exposure to renal ischemia caused damage to the tubules, but also that more severe damage was seen after the reperfusion. During the

exposure and reperfusion, a co-incubation with potential protective compounds was performed. One of the compounds – adenosine – showed strong protective capabilities with tubules nearly completely recovering, whereas the control without adenosine was highly damaged after the reperfusion period. The protective effect of adenosine was also observed in an *in vivo* study by Lee and Emala [31]. Here, rats were preconditioned with adenosine before hypoxia exposure and reperfusion, which was favorable for renal function and morphology. In addition, Lee and Emala investigated which of the adenosine receptors (AR) were involved in this effect. By administering AR agonists and antagonists, they discovered that AR1 is most likely involved in protecting against ischemic AKI. An interesting follow-up study would be to analyze the gene expression in our kidney-on-a-chip model, to see if it can be confirmed that AR1 signaling is the pathway which is responsible for protecting against ischemic AKI.

The most severe scenario of rIRI is when perfusion is stopped completely, radically reducing nutrient and glucose supply, and applying low oxygen. But it is also possible to test one of these conditions separately. Hypoxia for example can be caused by multiple events which lower the oxygen concentration in the blood. Hypoxia is associated with moderate-to-severe pneumonia which can be a result of an infection with a respiratory virus like the novel acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [32]. Here the platform could possibly be used to study compounds which have a potential protective effect. Our kidney-on-a-chip platform which we developed in **chapter 2** was already recognized as a potential platform to study AKI associated with SARS-CoV-2 [33]. The results obtained in **chapter 5** suggest that the use of the kidney-on-a-chip platform for these types of research could be feasible on short term.

Adding more complexity: Cocultures of the proximal tubule

In **chapter 5** the development of a coculture of proximal tubule cells with endothelium is described. For future investigations of the drug safety, cocultures will play a crucial role during the development of test models. The developed model could nicely show the suitability of even a quite simple model using a RPTEC cell line in combination with Human umbilical vein endothelial cells (HUVECs). Both cell types are not the perfect cell types to be used for the end model. For future optimization it would be better to use more relevant proximal tubule cells in combination with endothelium from the kidney. When the model was set up in 2017, endothelial cells from the kidney were used. Unfortunately, these cells did not form vessel structures and were therefore not sufficient to be used for setting up the ischemia model. With more companies offering new cell lines, among others induced pluripotent stem cells (iPSc), new cell sources will bring the current model to a new stage of predicting nephrotoxicity.

The combination of the kidney model with other organs like the liver or the gut system will likely be an important research area in the future. These organs also play an important role in the elimination of waste products from the body. Several systems can be studied: systems which offer the possibility to connect the different organ systems with each other, so-called body-on-a-chip

systems, or even simpler systems which are cultured in parallel and where only the conditioned medium is exchanged.

Future directions for model optimization

Optimization of the proximal tubule-on-a-chip using a more physiologically-relevant cell type

For this thesis two renal proximal cell types were used – RPTEC from Sigma-Aldrich and ciPTEC-OAT1 developed at Radboud University in Nijmegen. Only the combination of both cell lines was an adequate model for the validation of the nephroscreen model. RPTEC from Sigma are a pseudo-immortalized human kidney proximal tubule cell line (SA7K clone) generated via zinc finger nuclease-mediated knockout of a cell cycle protein [34]. ciPTEC are proximal tubule cells which were derived from human urine. These cells contain the temperature-sensitive vector SV40tsA58, allowing the cells to proliferate at 33 °C and differentiate at 37 °C [35]. This cell model was further optimized by Nieskens et al. [23] who were able to establish two additional cell lines from ciPTEC which express stable xenobiotic transporters organic anion transporter 1 and 3 (OAT1 and OAT3). OAT1 and OAT3 are the most abundant influx transporters of the proximal tubules mediating the anion transport into the pre-urine. When we compared the gene expression levels of the OAT1 expression, expression RPTEC was close to the detection limit and expression of OAT1 in ciPTEC-OAT1 was more than 150 times higher than in RPTEC . However, when ciPTEC-OAT1 are cultured in the OrganoPlate against collagen 1 cells do not form a barrier against the ECM which makes them unsuitable for use in barrier assessment.

In Table 1 available cell types and cell sources which have a potential to be cultured in the OrganoPlate similarly to the RPTEC and ciPTEC-OAT1 are summarized. For seeding cells to the 3-lane system it is of importance that the cells are added as a single cell solution to the perfusion channel. In the following paragraphs some of the most promising cell sources for future models are described in more detail.

In collaboration with the Hubrecht Institute tubuloid-derived adult stem cells from mouse kidney organoids were cultured in the OrganoPlate 3-lane system [46]. It could be shown that these cells form polarized tubular structures with clear distinction of the apical and basal side of the cells. Barrier formation of the cells against the ECM was confirmed by perfusing the lumen of the tubes with dextran dyes added to the culture medium [18]. We assessed transport function of the formed tubuloids on a chip using the assay developed in **chapter 2** of this thesis by exposing the cells to calcein-AM in presence or absence of PSC833, a non-immunosuppressant cyclosporine analogue that functionally inhibits P-gp [47]. Intracellular accumulation of calcein was significantly higher in the presence of PSC833, demonstrating activity of P-gp [46].

Table 1: Cell types and cell sources that are available for modeling of (future) Kidney-on-a-Chip models and their pros and cons, including some examples (content of table partly adapted from [36])

Cell type	Pros	Cons	Examples	References
Primary cells	*In vivo* morphology of the tissue source is usually maintained Allows donor specific analysis (personalized toxicity prediction) Easy to isolate Compatible with high-throughput screening and advanced imaging techniques	Differences between donors Limited availability Low throughput (because of limited lifespan) Inter-donor variability Functional changes during passages	Primary RPTEC	[37]–[39]
(Immortalized) Cell lines	High proliferation rate High reproducibility (homogenous population) Ease of use Decent amount of data available Cost-effective	Crucial transporters are absent Do not represent the complete spectrum of proximal tubule characteristics	RPTEC (SAK7 clone), HK-2, HEK 293, ciPTEC OAT1, ciPTEC-OAt3, RPTEC/TERT1	[23], [34], [40]–[44]
Human iPSCs	Easy to isolate Allows donor specific analysis (personalized toxicity prediction) Disease-specific *in vitro* cell-based models Expression of (all) relevant drug transporters?	Not completely characterized Complexity of culture protocol Genetic instability during long culture periods	iPSC differentiated into human primary renal proximal tubular cells	[45]
Derived adult stem cells from organoids	Allows donor specific analysis (personalized toxicity prediction) Direct expansion of patient-derived renal tissue	Heterogenous population (proximal, distal, loop of Henle, collecting duct) Culture conditions usually biased for one cell population (e.g. proximal tubule)	Tubuloid-derived adult stem cells from mouse kidney organoids	[46]

The trans-epithelial transport function of the cells could be demonstrated using rhodamine 123, which is transported into the cells from the basal side by organic cation transporters [48] and released to the lumen by P-gp [49]. Rhodamine 123 was added to the basal side of the tubes in the presence or absence of the P-gp inhibitor PSC833. In presence of PSC833 the apparent permeability was reduced significantly, suggesting a P-gp dependent efflux inhibition of rhodamine 123. Next to using cell lines with defined characteristics like the above introduced RPTEC, this model has a high potential to also be used with pluripotent stem cell-derived organoids, which would allow personalized studies on transporter-function and drug-interaction studies [46] and ultimately replace the RPTEC cell line in the future.

Recently a new promising cell source of RPTEC got commercially available on the market – RPTEC-tert1 [42], [44]. In addition to the parent version of the cells ATCC offers three modified versions which either overexpress transporter genes OAT1, OAT3, or OCT2. The parent version of RPTEC-tert1 was already used in a kidney-on-a-chip platform with promising results resulting in confluent tubules growing tight barriers against an ECM [50]. This makes them an interesting cell source to test in the OrganoPlate.

The market of renal proximal tubule cells is slowly growing and offering various newly developed proximal cell tubules sources. First studies by K. Kandasamy *et al.* [45] which use human iPSC from the kidney suggest a higher accuracy in predicting nephrotoxicity induced by drugs compared with using primary human proximal tubule cells.

In conclusion, we can say that new proximal tubule cell sources have a high potential. They may be of assistance to further develop the proximal tubule-on-a-chip model which to its end can reliably predict renal toxicity and can be used for a broad variety of pre-clinical drug studies and ultimately for complex disease studies.

ECM composition investigation of the proximal tubule-on-a-chip

3D cell cultures require an environment which is as similar as possible to the *in vivo* situation. This environment can be modeled on a huge number of different 3D culture platforms. In the last 15 years the number of publications describing 3D systems grew rapidly [51]. However, the first studies on 3D culture were already conducted 50-60 years ago, as summarized by Mina Bissell in 1982 [52] who was one of the first researchers studying the importance of the ECM on the cell behavior in *in vitro* systems. What all of these culture platforms have in common is that they all make use of an ECM environment. The ECM in the human body is a non-cellular component, which serves as a scaffold for diversity of different cell structures but also plays a huge role in the tissue morphogenesis, differentiation and homeostasis [53]. The ECM is comprised of water, proteins, and polysaccharides. However, the composition of the ECM is unique for each tissue. In the tubulointerstitium of the kidney collagen type I and collagen type III are the most abundant proteins, whereas in the basement membrane of the proximal tubule collagen type IV and laminin

are predominant, which networks are connected via perlecan and nidogen [54], [55]. Collagen of the interstitial space is produced by fibroblasts [56], whereas the basal laminar is produced by the epithelial cells themselves [55]. With fluorescent immunohistochemical stainings we could show that our RPTEC model was positive for the basal membrane proteins laminin, perlecan, and nidogen. This suggests that the basement membrane is indeed remodeled by the cells (figure 2). When developing our kidney on a chip model which was eventually used in thesis, we ended up successfully using an ECM gel made from rat tail collagen 1. We could show that collagen 1 imitates the tubulointerstitium of the kidney quite accurately. Here, it could be of high interest to investigate if an addition of collagen type III to the mixture would offer advantages. Another important aspect to consider when using ECM is their origin. All ECM components which we used originated from animal material. Therefore, future studies on animal-free ECMs are highly recommended. For general research synthetic ECMs could be a solution to overcome batch to batch variances and pave the way for standardized and reproducible compound investigations.

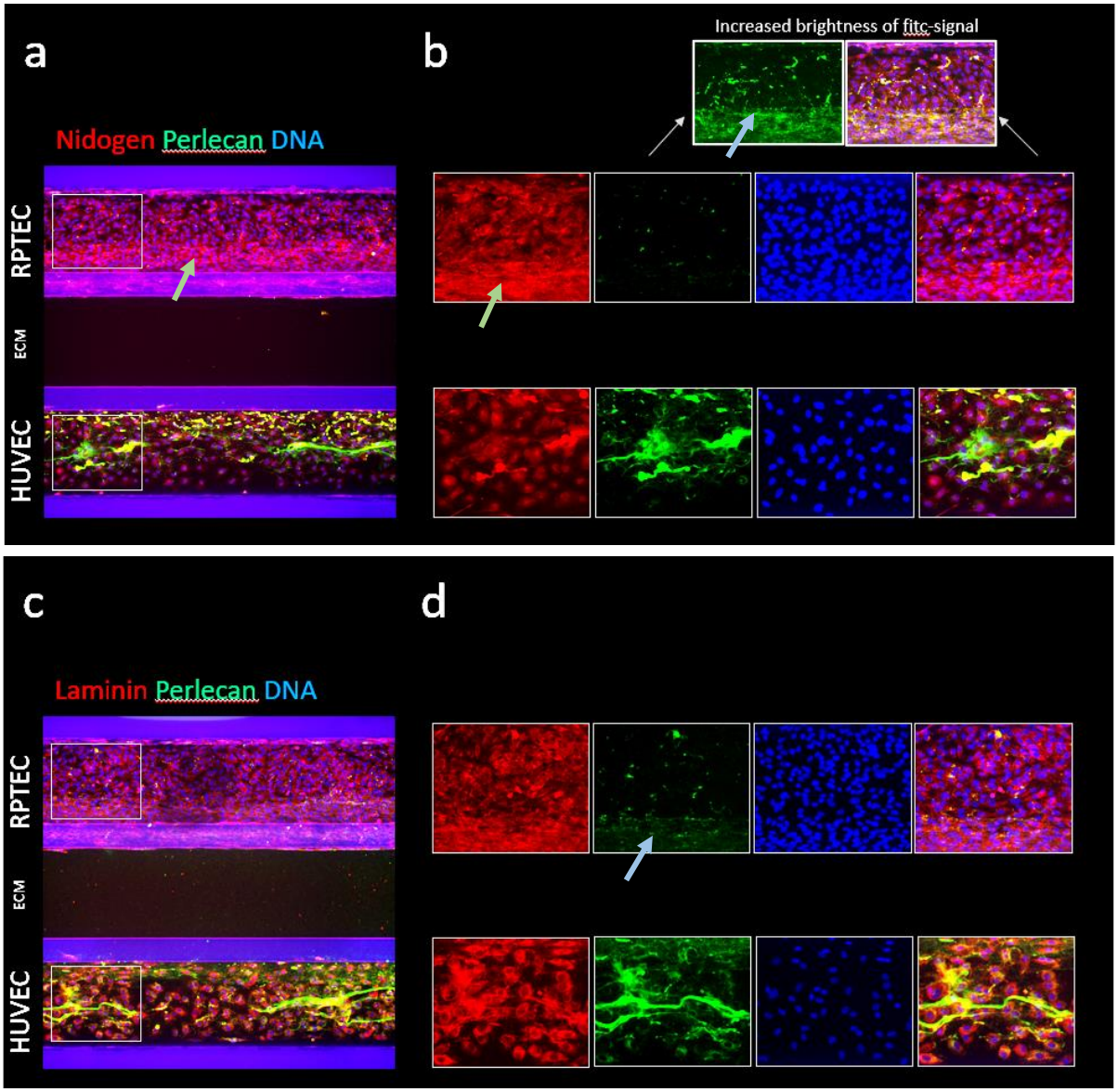

Figure 2: ***Marker expression of the kidney model shows presence of basal membrane proteins laminin, nidogen and perlecan of the coculture of RPTEC and HUVEC. A, C*** *z-projections of the coculture with the RPTEC tubule in the top channel and the HUVEC vessel in the bottom channel.* ***B, D*** *Zoom-in of the z-projections.* ***A, B*** *Nidogen (red) is expressed by both cell types, though the expression is more dominant in the epithelium with a higher expression at the cell/ECM interface.* ***A-C*** *Perlecan (green) expression is significantly higher expressed in HUVEC, though this appearance is dominated by strings. These strings appeared to be atypical staining. Perlecan was less expressed by the epithelial cells than endothelial cells. Expression is almost exclusively found at the cell/ECM interface with an even higher difference compared to Nidogen.* ***A, C*** *Laminin (red) is expressed equally by both cell types, with no significantly higher expression at the cell/ECM interface.*

The use of human derived ECMs could open possibilities to study donor-specific medical conditions, for instance matrix metalloproteinases which are suggested to play a role in tubulointerstitial fibrosis [57].

Conclusion

Pharmaceutical companies, governments and the general public have become increasingly aware that animal models used in drug testing lack some vital aspects in the endeavor to serve as an accurate representation of human biology. As these models of the human body should be more physiologically relevant, animal models no longer suffice because the response of animal cells often differs from the response of human cells. In their place, *in vitro* cell culture models with 3D architecture, microfluidics and high throughput capabilities are a promising technology and are currently getting in the limelight of drug research. These 3D models can be developed in such ways that they will likely surpass animal models on important aspects like resemblance to a human body, predicting safety and efficacy of compounds, high throughput testing capabilities, ethical aspects, and costs.

To demonstrate the feasibility of such an advanced 3D invitro model, we used a microfluidic *in vitro* platform to develop a kidney-on-a-chip platform which possess the ability to reproduce the tubular response to known and unknown nephrotoxicants and compounds as seen in *in vitro* and in clinical studies. Furthermore, we assessed the response of the model to renal ischemia/reperfusion injury and could measure the prevention of tubular damage when adding protective compounds.

These findings show that 3D tissue models are able to compete with alternatives like animal models and 2D models. We actually expect that 3D tissue models are the test platforms of the future for developing new drugs.

Since research on 3D tissue models is a relatively new research field there remains a large scope for improvement. For example, the endeavor to use *in vitro* findings to confidently predict *in vivo* behavior is an area of research where 3D tissue models can play an important role. Ways of replicating different human organs on 3D chips are likely to greatly improve in the coming years, combining different chips and creating interactions between them can test the effectiveness of drugs at a whole new level. In this way the complexity of the *in vitro* 3D models can be increased, in ways which better resemble tissue responses in humans.

This could lead to testing a drug simultaneously on different organs while connecting them in ways resembling real structures in the human body and measuring responses of each different organ singularly. When these 3D tissue models are cultured in a high throughput environment huge amounts of data can be produced in short time. These data can serve as a foundation for mechanistic models using big data approaches that can be ultimately used to rapidly predict *in vivo* drug efficacy and safety.

Interesting examples of complex future applications of 3D tissue models are for instance complete *in vitro* vascularized organoids, ‘body-on-a-chip’ systems which can be designed for a specific test

to combine all relevant tissues and their interactions in order to resemble *in vivo* responses. Another example is the creation of complex disease models where the response of tissue and their interactions can be tested in a realistic way. Another potential future application of 3D tissue models is personalized medicine, where a high number of replicates of patient cell samples can be cultured in 3D on the microfluidic chips to create patient derived *in vitro* models. These cell samples can be subsequently screened for the most effective treatment for this individual patient using his own cells. In time, the further development of 3D tissue models could mean that these examples are no longer dreams but become reality.

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