Steps toward pre-clinical iPSC-derived kindey organoids

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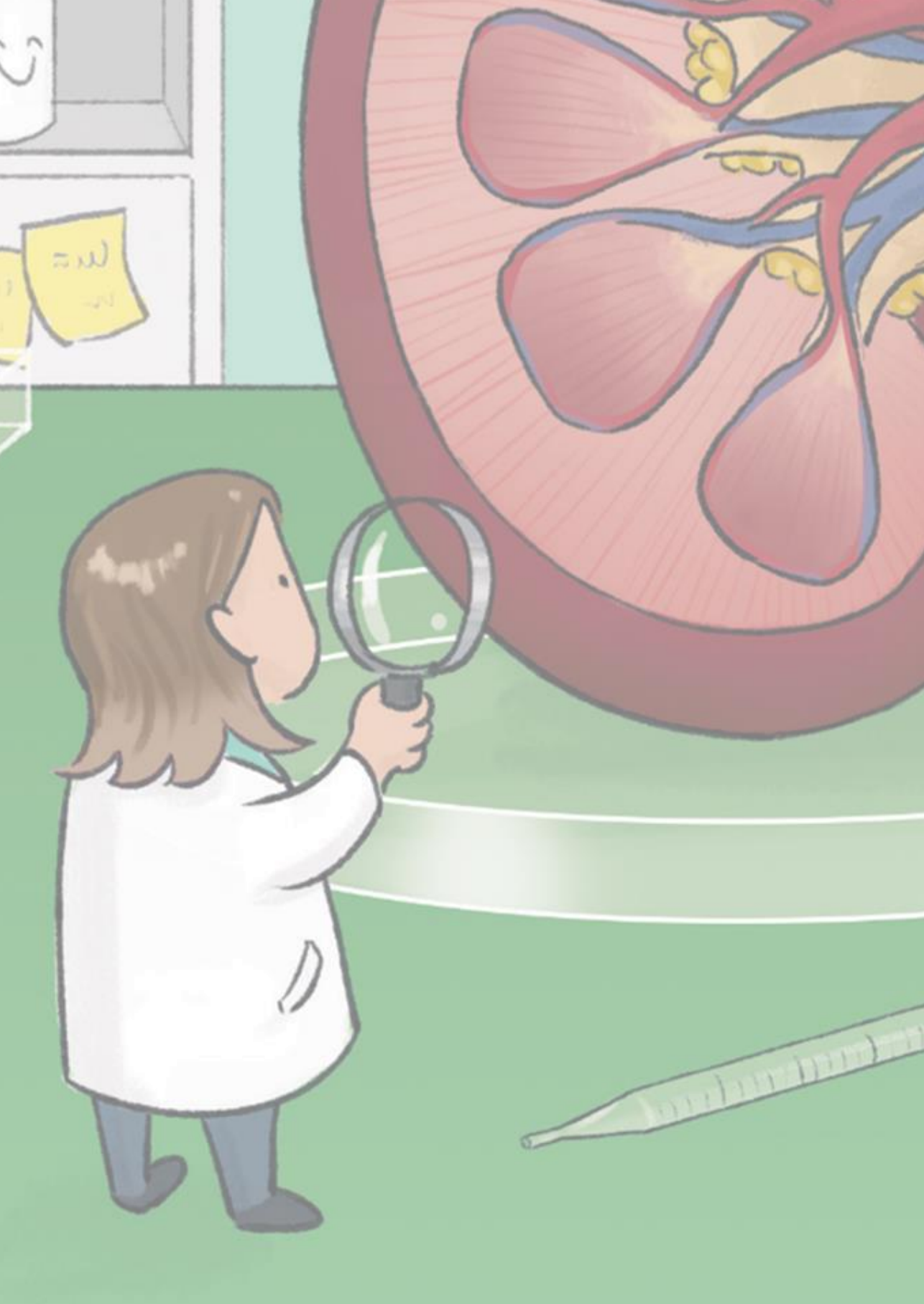

Chapter 6

General Discussion

Organoids are self-organizing, three dimensional tissue cultures that contain structures which mimic human organs, and represent a significant improvement beyond traditional two dimensional cell cultures. Research in this rapidly advancing field, specifically related to the kidney for the purposes of this thesis, has demonstrated that kidney organoids hold great potential in regenerative medicine, drug screening, and developmental biology [1-3]. They are also considered for the field of organ transplantation as a potential source of transplantable tissue. However nephron immaturity, low throughput, limited scale, lack of vascularization, off-target populations, reproducibility and replicability hinder the current application of these kidney organoids [4-7]. In this thesis we present an explorative analysis of different aspects to advance these kidney organoids for the generation of auxiliary kidney tissue.

Scalability

One of the primary challenges in applying kidney organoid tissue for clinical use is their limited size. Kidney organoids usually range up to 3-4 mm in width and contain a few thousand nephron structures. This number was determined through 3D imaging and by calculating the number of glomerular structures throughout the organoid. To generate an adequate number of nephrons to result in an effect on human level, scale-out, a method to increase the number of small organoids [8-10], or scale-up by increasing the size of the organoid ([11] and **Chapter 2**) are of great interest. In the newly developed nephron sheets in **Chapter 2**, we estimated tens of thousands of glomerular structures per sheet, with some variation per cell line. To compare, in humans there are approximately 1 million nephrons per kidney. Therefore, these nephron sheets could present an opportunity to postpone or diminish the need for dialysis or kidney transplantation, provided that all nephron structures in a sheet become fully functional upon transplantation and are as efficient as regular nephrons. To put this into perspective; dialysis replaces approximately 15% of kidney filtration, and 4 nephron sheets reach a comparable 15% nephron count. Similarly, Lawlor et al have explored manipulation of kidney organoid shape through automated printing [11]. Printing proved to be faster and more accurate than manual pipetting and was also able to create a large organoid patch. This data shows that the organoid protocol is flexible and, to a degree, able to be used in a system that gears towards automation of cell culture and differentiation processes. In the future, integration of such protocols for scale-up and scale-out could set up a kidney organoid differentiation platform combined with automated maintenance of iPSCs, which could hold great merit for clinical purposes.

Qualitative assessment

In **Chapter 3** we discuss the importance of qualitative assessment of kidney organoids. For these tissues to be used in clinal translation they must be save and produced under good manufacturing practice (GMP) compliant manufacturing protocols to ensure quality. Protocols for generating iPSCs under GMP compliant procedures have recently been published [12,13]. Currently there are no GMP compliant procedures for generating kidney organoids, but in **Chapter 3** we discussed the use of specific quality assessments to screen kidney organoids. First, the viability of kidney organoid progenitors on day 7 of differentiation both for non-cryopreserved and cryopreserved progenitors needs to be above 90 %. Second, we propose the utilization of advanced Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry Imaging (MSI) to assess the quality of the kidney organoids. MALDI-MSI takes images over hundreds of molecules unbiased in intact fixed tissues and assess subtle changes in analytes to allow for unbiased comparisons between different tissues [14]. With MALDI-MSI we were able to compare organoids from non-cryopreserved and cryopreserved progenitors, and found organoids from either origin contained the same type, number and relative size of kidney related populations. Kidney organoids still have a batch-to-batch variability which has been observed previously [15]. This indicates that the organoids within a differentiation are relatively similar but there is a greater variety between batches. Prospectively, the cryopreservation step could also be implemented in an automated kidney organoid technology platform. Automation of the organoid differentiation and cryopreservation process might aid to reduce the observed variations. Of course there are many other facets of the kidney organoid to take into account during quality assessment. For instance by looking at different (physiological) features, metabolism, and functional assays, with the intent to produce a high-quality organoid that is safe for research and clinical application [6].

Maturation and filtration

An objective potential of kidney organoids for clinical use lies in their ability to be transplanted, thereby enhancing filtration capacity in patients with chronic kidney disease (CKD). Transplantation of these organoids in mice and chicken embryos has shown integration of the organoid with the vasculature of the host, enhanced nephron maturation, and (size-selective) filtration of dextran molecules [16-20]. In **Chapter 4** we explored the morphological development of each specific nephron segment of kidney organoids after transplantation by using electron microscopy with high spatial resolution imaging. This ultrastructural characterization enables analysis of the morphological appearance of the cell, offering valuable insight into their functional efficacy. This relates to the formation of the glomerular filtration barrier, microvilli on the proximal tubule, and cell type specification in connecting tubule. Morphological characterization can be challenging, especially in young

tissue as many of the identifying features have not developed at a younger stage. However after 4 weeks of transplantation fenestrated endothelium, tri-laminar glomerular basement membrane and podocytes with foot processes could be observed in the glomerular structure. We also observed well-developed microvilli in the proximal tubule, developing Loop of Henle and/or distal tubules, and connecting tubules with different specialized cells. These observations on functional morphological development was thereby suitably shown.

The presence of a vascular network in the kidney organoid has been shown after transplantation, and progress to achieve this in vitro has been made. The capillaries of this network can sustain the kidney structures of the organoid, but also create the vital connection to the glomerular structures to form the glomerular filtration barrier. Organoids in vitro spontaneously generate some endothelial cells but these are lost overtime. Organoids on a millifluidic chip under flow have shown a significantly higher number of endothelial cells [21]. Alternatively, combining genetically inducible ETS translocation variant 2 iPSCs (iETV2-hiPSC) with naïve iPSCs have shown to generate a kidney organoid with a high number of endothelial cells that encase and invaginate podocyte populations [22] in vitro. In both papers the organoid also achieved a more matured status. The endothelization and vascularization of the kidney organoid is highly important seeing that the kidney receives approximately a quarter of the cardiac output [23]. Vascularization in vitro can provide a more accurate kidney organoid for drug testing by either the manner of drug administration or the developmental state of the organoid. For transplantation it might also prove beneficial to achieve a faster connection to the host, creating less stress to the tissues and saving nephron structures that might be lost due to lack of nutrients caused by the delay in vascularization.

Reaching a higher state of kidney organoid maturation and striving for complete filtration, the outlet of the filtrate generated by kidney organoid becomes an important topic. Therefore the ability of PSCs to differentiate into derivates of the ureteric bud (UB), with the ability of inducing branching morphogenesis, is an important goal. Kidney organoids in this thesis mainly contain derivatives of the metanephric mesenchyme to generate segments of the nephron. However there is a lack of the collecting duct and further differentiation of the ureter that lead towards the bladder for a filtrate outlet. It has been proposed to combine the nephron, stromal, endothelial and collecting duct progenitors [24-26], however creating a collective receptacle like a ureter or bladder poses difficulties [27]. This is highlighted by the current patterning of the organoid nephron structures as it does not uphold a clear separation like the medulla and cortex in the kidney [28].

UB organoids from hPSC that mimic branching and develop to a collecting duct have been generated [29], and combining UB and metanephric mesenchyme (MM) progenitors has shown a more matured kidney organoid with fewer off-target populations [24,25]. Kidney-specific epithelial parenchymal cells, called stroma, have been argued to play an important

role in the kidney's development [30,31]. Combining stromal cells, UB and MM progenitors from mouse embryonic stem cells (ESCs) to form an organoid showed improved branching [32], however this has not been done with hPSC. The induced stroma was able to differentiate into cortical and medullary stromal cells, as well as mesangial cells and renin cells. This is an important step towards creating a more complete kidney organoid with a higher order structure containing NP and UB derivatives, however the lack of a urinary outlet remained. Further stimulation of UB progenitors could potentially lead to the formation of a unified filtrate outlet to improve the ability of the organoid to mimic native kidney drainage. Alternatively, advancements in transplantation strategies, such as optimizing the implantation site or enhancing integration with the host, may also provide a solution.

Functionality of on-target cells

Next to filtration, other functions of the nephron structures in kidney organoids have been investigated as the kidney (interstitium) is also responsible for the production or activation of various homeostatic hormones [33]. Shankar et al. have demonstrated that the organoids are capable of metabolizing vitamin D into its active form [34] which is a physiological role of the kidney in vivo, as well as the secretion of the hormone renin by the stromal cells of kidney organoids [32,35]. Renin is a hormone that affects blood pressure and is secreted by the juxtaglomerular apparatus, which is a part of the kidney located between the afferent and efferent glomerular capillaries. However if renin is produced by cells of the juxtaglomerular lineage in kidney organoids is yet unclear. Next to this, functionality of, and expression of (mature) receptors in the (proximal) tubules is displayed by uptake of dextran. This has been shown in tubules of the kidney organoid upon transplantation [17,18](**Chapter 2)**.

Reduction off-target cells

Next to these advancements, we have also observed a safety concern in these kidney organoids: they contain off-target cell types which could be potentially harmful [4,20,36-38]. The number off-target cells does appear to lower after transplantation [20,38] compared to in vitro. Cartilage is one of these off target cell populations hindering further research both in vitro and in vivo. The origin of this off-target population remains unknown and there is no clear overlap between the kidney organoid and cartilage protocol to readily explain its development. In **Chapter 5** we explored the use of TGFβ blocking agent SB431542 to inhibit this in kidney organoid development. The TGFβ superfamily is known to be involved in cartilage development [39,40], and 5 μM SB431542 proved to be an effective dose to significantly reduce the appearance of cartilage whilst maintaining the nephron

structures. Although cartilage was not completely suppressed, the results are encouraging. Further downstream investigations could be valuable in developing an effective protocol to prevent cartilage development in kidney organoids. Furthermore, MALDI-MSI, single cell RNA sequencing or spatial transcriptomics could hold great merit to identify the underlying cause of such off-target population. When identified these markers should be taken along in the aforementioned quality assessment to ensure proper safety guidelines for the use of kidney organoid for transplantation [41-44].

Concluding and future perspectives

This thesis explores the use of iPSC-derived kidney organoids in human medicine, focusing on size manipulation, the ability to cryopreserve kidney organoid progenitors, and the potential of MALDI-MSI as a quality assessment tool. Additionally, the morphological maturation of the organoid was investigated upon transplantation and removal of off-target cells. See Figure 1 for a summary overview of this thesis.

The future and potential of kidney organoids holds many interesting promises and subsequent research should focus on kidney organoid protocol optimization to eliminate the presence of interruptive off-targets as it could open a path to further research both in vitro and in vivo. Transplantation studies could be further optimized by including endothelial cells and improving maturation of kidney organoids in vitro. Optimization of the (micro)environment of the kidney organoid, like organ on a chip and bioprinting, could further enhance the organoid in vitro [45], or improving nutritional requirement for iPSCs and kidney organoids to allow for proper maintenance and development [14,46]. Furthermore, robotic systems and bioprinting platforms can help to minimize batch-to-batch variations and the ability to cryopreserve allows for the generation of biobanks that could assist in reproducibility of experiments.

Organoids still represent foreign body tissue and can thereby elicit a strong immune response after transplantation due to the activation of the adaptive and innate immune system [37,47,48]. To combat this, gene-editing to generate hypoimmunogenic hPSC that can evade the immune system by knocking out specific immune related genes are currently investigated [49]. Knock-out of beta-2 microglobulin in iPSCs using CRISPR-Cas9, resulted in an iPSC line that lacks HLA class I expression [50]. However, this knockout did not protect the kidney organoid against T-cell-mediated immune rejection in vivo. Importantly, it has

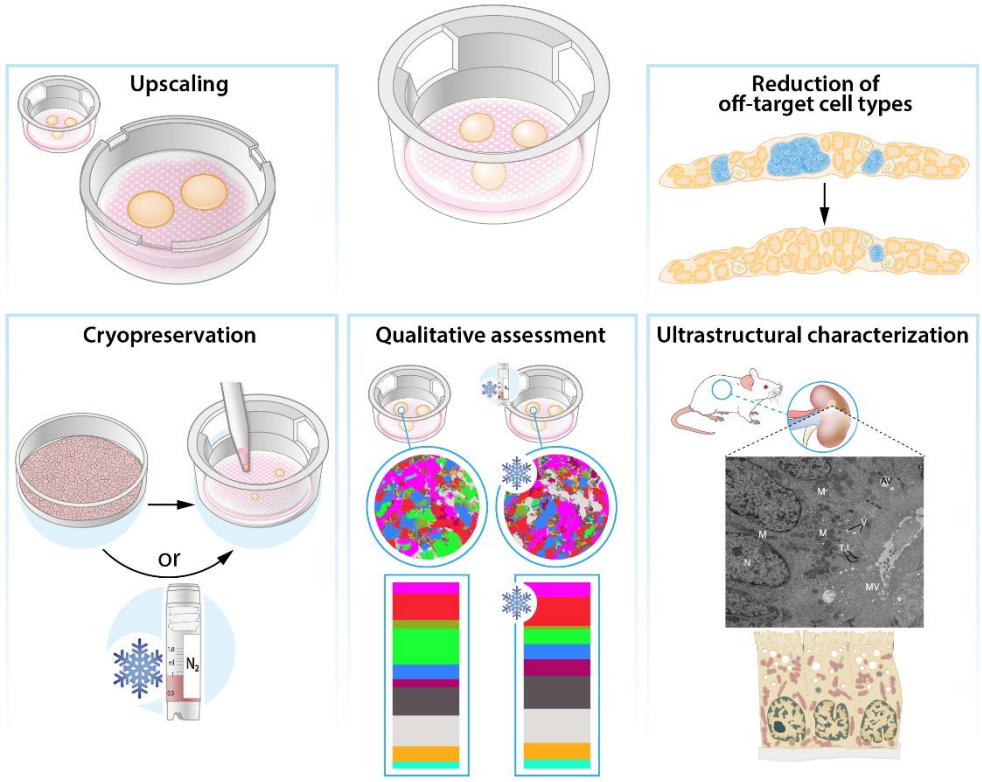

Figure 1. Subject summary overview of this thesis

also been demonstrated that transplantation of beta-2 microglobulin-knockout in hESCs can activate natural killer cells [51]. Overexpression of complement inhibitor CD55 in PSCs gave protection from an antibody mediated immune response [52]. Other methods that alter the expression HLA class I, and simultaneously increase or decrease other receptor expressions are being investigated to generate an immunogenic PSC line [49,53]. The topic of organoid rejection, similar to organ rejection, is of major importance and the use of such immune-evasive hPSCs also demands rigorous safety measures.

To summarize; while patient-derived organoids hold promise for personalized medicine - like the use as a transplantable tissue, key challenges remain. The process of kidney organoid induction, maturation, vascularization, quality assessment and safety still require extensive research. Focus on accurate assessment methods during organoid development may lead to a more reliable protocol that has a higher probability of well-developed organoids, increasing safety, and thereby creating an opportunity to study long term effects of transplantation. Ongoing advancements in technologies such as genome editing, single-cell RNA sequencing, MALDI-MSI, hydrogels, bioreactors, printers, and milli/ microfluidic chips are continuously refining the field. Combined with new scientific insights, these

innovations will enable organoid technology to grow towards a safe and functioning transplantable tissue.

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