Steps toward pre-clinical iPSC-derived kindey organoids

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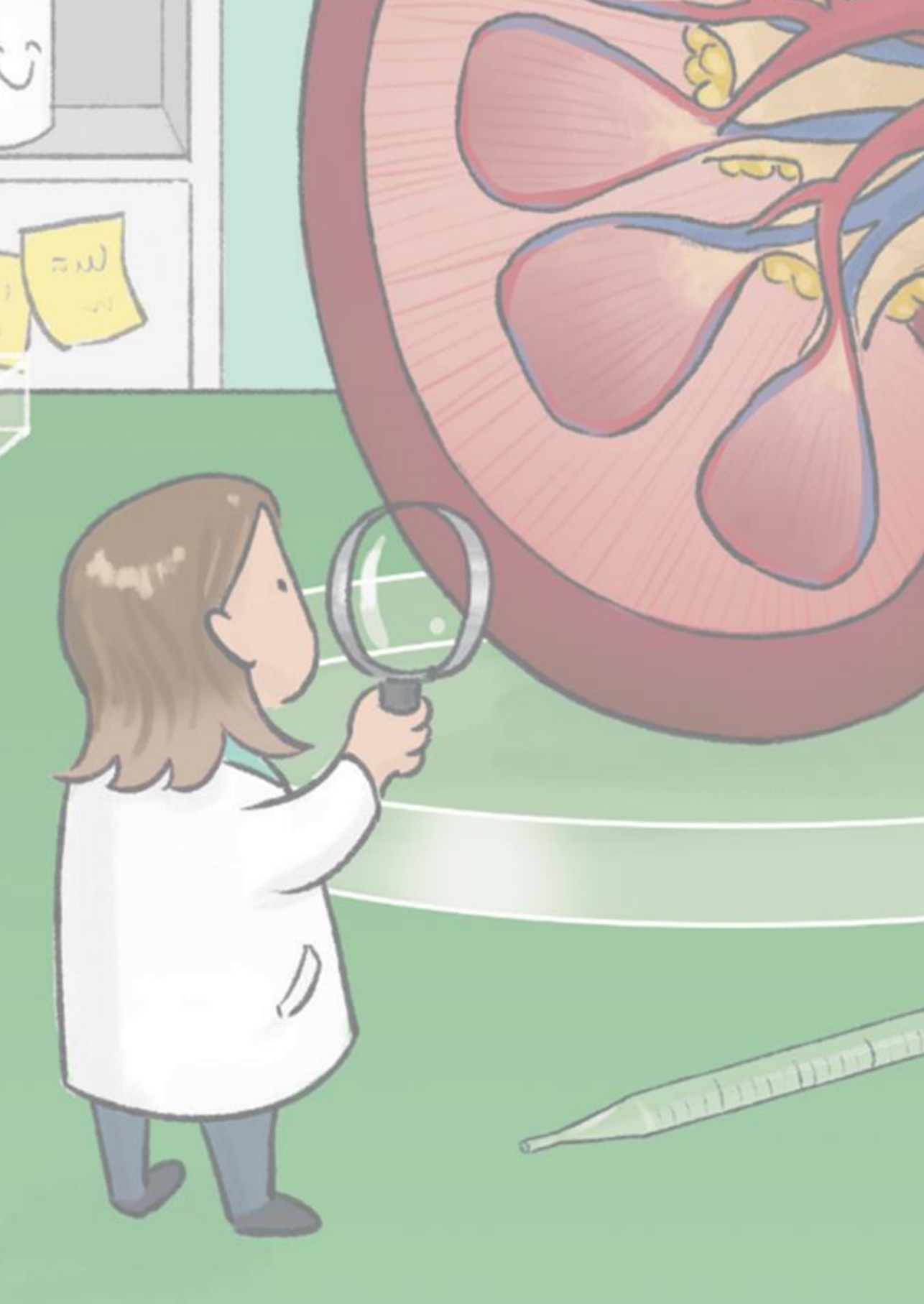

Chapter 1

General introduction and outline of this thesis

Kidney function and disease

Kidneys are complex and intricate organs that play a vital role in maintaining the body's homeostasis. They regulate water and electrolyte balance, blood pressure, specific hormones, and filter waste from the blood for excretion via the urine [1]. Effective filtration depends on the proper development of the kidney's nephron structures. These nephrons are the filtering units of the kidney and each kidney contains approximately 1 million of these nephrons. In humans, nephrogenesis exclusively occurs prenatally, with complete maturation occurring postnatally. Nephrons consist of distinct segments, beginning with the glomerulus encased by the Bowman's capsule. The glomerular structure consists of a filtration barrier formed by specialized epithelial podocytes that wrap around the glomerular capillaries, allowing selective passage of small molecules while keeping larger molecules in the blood. Filtrate collects in the Bowman's space and continues to the proximal tubules, which are responsible for a significant portion of the reabsorption. Next, the filtrate flows through the loop of Henle, which primarily reabsorbs water and sodium chloride. The distal tubule follows the loop of Henle and regulates extracellular fluid volume and electrolyte homeostasis, before leading to the connecting tubule and collecting duct which play a vital role in finalizing the filtrate before entry into the bladder [2-7]. See Figure 1 for a schematic overview of a nephron.

It is estimated that 10% of the world's population is affected by kidney disease [8]. Acute kidney injury is defined as a rapid, potentially reversible, decline in renal function caused by disease, injury or medication. Alternatively, chronic kidney injury is a progressive deterioration of renal function. It is estimated that 697 million people have chronic kidney disease (CKD) globally [9], with the number steadily rising [8,10] as we live in a world with a growing population with increased age, obesity, diabetes and cardiovascular diseases [11]. CKD can be divided into five stages based on the glomerular filtrate rate (GFR). A normal GFR is ~ 90 mL/min (stage 1) that degrades to end stage CKD (stage 5) with a GFR rate lower than 15 mL/min (also referred to as end-stage renal disease (ESRD)). Treatment of ESRD is restricted to dialysis or kidney transplantation. Dialysis is a life sustaining treatment that replicates the filtering function of the kidney to remove waste, excess fluids, and toxins from the blood, but this procedure is burdensome for patients as it involves frequent visits to the hospital. A preferred alternative is transplantation with a donor kidney, but there is a shortage of available donor organs. Recipients have an increased quality of life, with fewer dietary restrictions, but need to take immunosuppressive drugs and there is a risk of donor organ rejection .

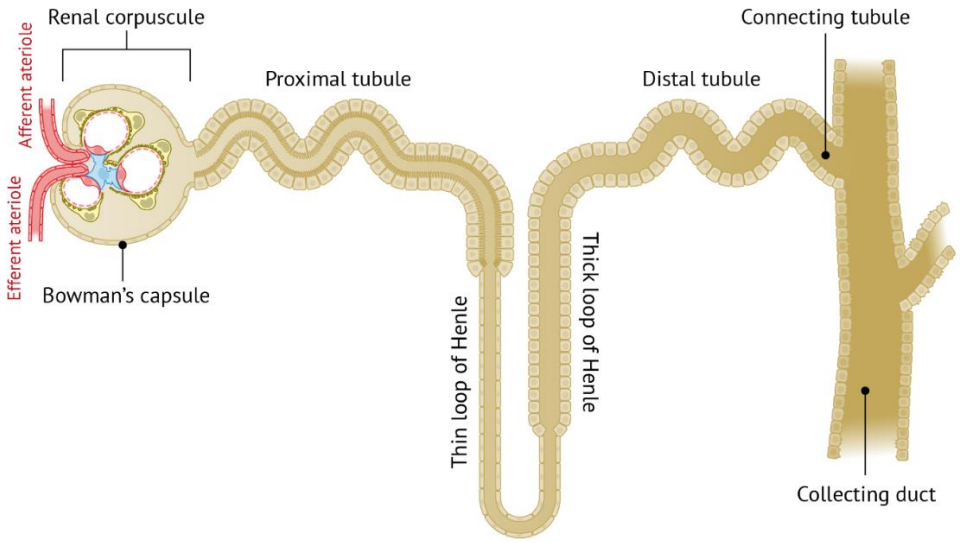

Figure 1. Schematic overview nephron

Regenerative medicine and (pluripotent) stem cells

Due to the limitations of current renal replacement therapies it is important to look for alternative treatments. Regenerative medicine is the field of biomedical research that focuses on repairing, replacing, regenerating, and or enhancing the function of damaged tissue with the goal to develop innovative therapies. Research areas of interest therefore focus on generating new organ tissue by tissue engineering and developing cellular treatments.

Stem cells have the ability to differentiate into various specialized cells. Somatic, also known as adult stem cells, have a restricted differentiation ability and are present in adult tissues where they are essential for maintenance and repair. In contrast, embryonic stem cells are pluripotent and possess the unique ability to differentiate into nearly any cell type. However, these cells derive from the inner cell mass (ICM) of early-stage embryos and their use in research raises ethical debate. A breakthrough came in 2006 when mouse somatic cells were reprogrammed to pluripotent stem cells [12], soon followed by the first reprogramming of human somatic cells at the end of 2007 [13,14]. These reprogrammed cells are referred to as induced pluripotent stem cells (iPSCs). This revolutionized the application of pluripotent stem cells in research, as these cells could now be generated from easily accessible somatic cells, like blood cells or skin fibroblasts. These iPSCs hold great promise for regenerative medicine by enabling tissue engineering and the development of (personalized) cellular therapies. The challenges lie in generating functional tissues from iPSCs which requires precise protocols based on an understanding of organ development.

Kidney development and kidney organoid generation

After fertilization the zygote develops into the blastocyst, of which the ICM will continue embryological development by forming the endoderm, ectoderm, and mesoderm germ layers. The kidney develops from the intermediate mesoderm, and progresses through 3 stages: pronephros, mesonephros and metanephros. The former two are only seen during embryogenesis. The metanephros develops through intricate signaling between the metanephric mesenchyme (MM) and the ureteric bud (UB) that emerges from the Wolffian duct. Both are essential for development of the final kidney [15]. The signaling drives branching morphogenesis of the UB structures to form the collecting duct system, while the MM forms the nephrons. The developing nephron starts as renal vesicles, and progresses to the comma-shaped body, S-shaped body, and capillary loop nephron stages [15-19] to form a glomerulus with connected tubular structures. The glomerular structure generates a cleft in which endothelial cell progenitors migrate [1] to form the capillary network. This connection is critical for the formation of the filtration barrier. This understanding of kidney development is applied to develop protocols for generating kidney organoids from the iPSCs.

Organoids are multicellular, self-organizing tissues and mimic the architecture and cellular composition of their organ counterpart. Since the generation of iPSCs, many protocols to differentiate these cells to kidney organoids have been developed [20-26]. These protocols aim to recapitulate kidney development by differentiating PSCs first to mesoderm, followed by intermediate mesoderm, and continue to kidney precursors by applying specific growth factors. Despite following the same trajectory in nephron (precursor) development the protocols vary in culture where organoids can be grown on an air-liquid interface, submerged in a well, or in suspension culture. Most protocols show an overlap in the growth factors applied, such as Wnt-activators to form (intermediate) mesoderm and factors like FGF9 to direct these cells to the cells of interest, but their respective supplement administration, duration and complexity vary. In all, these protocols result in organoids that contain nephron structures and have been proposed as attractive tissues to develop therapies replacing or regenerating damaged tissue, thereby restoring function of these organs in renal disease patients. Kidney organoid protocols have improved over time, yet significant challenges remain for the clinical use of these organoids.

Current challenges of kidney organoids

Despite advancements in generating kidney organoids from iPSCs, further improvements are needed. For instance, the structures of the kidney organoid remain immature in vitro. The immaturity can be detected by the underdeveloped glomerular basement membrane, closed tubules, lack of specialized protein expression, and by RNA sequencing which matched the organoids to fetal kidneys in the first trimester [23]. Transplantation has shown

to enhance maturation, but there is a concern for safety and off-target growth, and the integration of organoid to host for filtrate excretion.

In vitro kidney organoids lack a vascular network and thereby do not have filtration capabilities. The absence of a working vasculature halts the formation of a proper glomerular filtration barrier, and the lack of flow through the tubular structures does not allow for further morphological development to take place. It is proposed that the introduction of a vasculature that connects to the glomerulus will initiate further maturation of the organoid in vitro. Endothelial cells have been observed in the organoid but due to the absence of (vascular) flow, they lack organization into a functional structure and diminish upon prolonged organoid culture. It has been demonstrated that culturing kidney organoid under flow on a millifluidic chip increases the presence of a capillary network in the organoid itself [27]. However this network does not establish active filtration. Transplantation of the kidney organoid does stimulate connection of the vasculature of the host to the blood vessels in the kidney organoid, which stimulates continued development and maturation of the organoids compared to in vitro organoids [28-32]. Live imaging of transplanted kidney organoids has shown size selective sieving by the glomerular structure, visualizing filtration into the Bowman's space [33]. This reveals functional maturation of both endothelial cells and the podocytes of the glomerular structure in the kidney organoid.

Another challenge is the formation of a collecting duct in the kidney organoid. The collecting duct is generated from the UB unlike the rest of the nephron, and is the final part following the distal and connecting tubule, where it finalizes the filtrate for excretion. Protocols to include the collecting duct in the kidney organoid are being investigated [34,35]. Furthermore, if the kidney organoid is to be a curative treatment in patients, it is vital that the tubular structures of the kidney organoid connect to the host tubules to clear the produced filtrate. This has yet to be established.

To effectively restore kidney function in patients, the relatively small kidney organoids need to reach the capacity to filter meaningful volumes of blood. For reference, dialysis achieves approximately 15% glomerular filtration and clearance rate. To be clinically impactful as replacement tissue or to delay the progression of CKD, kidney tissue generated in the lab needs to reach an equal or higher level of filtration. Scaling of the kidney organoid is challenging as thicker organoids struggle with nutrient delivery to the center and removal of toxins and waste. Therefore, scale up, increasing the size of the organoid, or scale out, increasing the production of organoids, is of main interest to achieve a stem cell derived tissue at a level that is suitable for therapeutic applications.

Beyond addressing the functionality and size of the organoid, it is crucial to translate laboratory protocols into clinically applicable protocols with stringent guidelines for quality and safety standards required for human medicine. It is important to set these quality

control guidelines for PSCs, progenitor populations during differentiation, and the kidney tissues ensuring the quality of each organoid. Safety standards are crucial as there is concern for stem cell derived tissues to form off-target cells that are not normally present in the tissue of interest, such as neural cells, muscle cells and chondrocytes [32,36-39]. Off-target cells can pose a danger if they were to become malignant and invade nearby functional cells, like chondrocytes that form cartilage which can suppress space for the functional on-target cells. Therefore we need to ensure that a transplanted kidney organoid remains safe and maintains function over time.

Outline

This thesis focuses on some of the current challenges that must be addressed before hu man iPSC derived kidney organoids could be proposed as an auxiliary replacement tissue. Organoid protocols tend to yield small structures, and these sizes are not sufficient to replace kidney function in patients. In **Chapter 2** we explore the ability to adapt the kidney organoid protocol to generate efficient larger-sized tissues with the same capabilities as their smaller counterpart. Simultaneously, the ability to cryopreserve the kidney organoid (precursors) is explored.

If the organoid is to be applied as a product, for instance in tissue replacement therapies, it must undergo qualitative assessment before it can be utilized. In **Chapter 3** we explore the use of a specialized technique, MALDI-MSI, as an in depth characterization analysis tool providing information on tissue composition. Here we compared cryopreserved and non-cryopreserved kidney organoids in vitro.

In **Chapter 4** we further investigated the maturation of kidney organoids after transplantation and emphasize the importance of the morphology of the different nephron structures using transmission electron microscopy. This technique facilitates detailed morphological analysis on cellular level, allowing us to explore the complex structural characteristics of these nephron segments.

To further analyze the kidney organoid after transplantation, extended studies are required. Currently off-target cell populations pose a challenge, as their presence increases over time, thereby raising concerns about functionality and safety. In **Chapter 5** we performed a preliminary investigation to screen if we are able to reduce the appearance of the off-target cartilage population.

Chapter 6 provides a summary of the research presented in this thesis and discusses future perspectives on application of kidney organoids for regenerative medicine.

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