Exploring kidney organoid vascularization

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CHAPTER 7

General discussion

End stage kidney disease has a major impact on the lives of patients as well as on healthcare costs. In the Netherlands, one year of dialysis for one patient costs 80.000-120.000 euros. The expenses of a kidney transplantation amount to 80.000 euros, after which the yearly costs are much lower than those for dialysis[1]. Due to the insufficient number of donor kidneys and the risks associated with the lifelong use of immunosuppression, there is an urgent need for innovative renal replacement therapies. Human induced pluripotent stem cell (hiPSC) derived kidney organoids provide an excellent base for the development of such strategies. **Chapter 2** describes the characteristics and potential of these organoids that contain nephron-like structures surrounded by stromal and scattered endothelial cells (ECs), and discusses important hurdles towards clinical application. As such, it introduces the main focus of this thesis: The lack of a functional vasculature and consequent immaturity of kidney organoids.

The kidney and its vasculature: an essential collaboration

The notion that the sole function of vasculature is the delivery of blood and nutrients to cells and tissues does not in the least do justice to the complex interaction of blood vessels and vascular ECs with their surroundings. The intricate and specialized renal vasculature is an excellent example of this symbiosis. Kidneys are burdened with the task of maintaining our fluid and electrolyte balance and removing waste products. They manage this by filtering 180L of water per day from the 1500L of blood that pass through them, allowing for passive filtration of waste products into the tubules. Through tubular reabsorption of 99% of the filtered water together with electrolytes and amino acids, around 1,8L of concentrated urine is produced and homeostasis is maintained[2]. The filtration of such large quantities of water is dependent on specialized fenestrated glomerular ECs. The post glomerular vasculature gives rise to peritubular capillaries that surround the proximal tubule, lis of Henle and distal tubules, supporting the reabsorption of water and essential solutes. Without blood vessels, the kidney is unable to execute any of these functions. In addition, the interaction between renal epithelial cells and ECs is essential for the development and maintenance of a mature kidney. During embryonic development, podocyte progenitors recruit ECs through the secretion of VEGF-A [3-5]. In turn, glomerular ECs secrete angiocrine factors including delta-like 4 (DLL4), Dickkopf-related protein 2 (DKK2), semaphorin-5A (SEMA5), VEGF-A and fibroblast growth factor 1 (FGF-1) that are implicated in the differentiation and maintenance of podocytes and mesangial cells [6,7]. Together, ECs and podocytes produce the glomerular basement membrane (GBM). Cross-talk between ECs and epithelial cells in the kidney is not limited to the glomerular structures. Proximal tubular epithelial cells stimulate ECs to secrete increased amounts of transforming growth factor β1 (TGFβ1), its antagonist α2-macroglobulin, hepatocyte growth factor (HGF) and VEGF, supporting the maintenance and proliferation of the tubular epithelial cells [7,8].

Organoid vascularization upon transplantation: the solution to our problems?

Considering the importance of the interaction between renal progenitors and ECs, it is not surprising that unvascularized kidney organoids lack maturity. Interestingly, they do have the potential to induce sprouting angiogenesis from existing blood vessels upon transplantation in mice[9-11]. To enable detailed analysis of the process of organoid vascularization, in **Chapter 4 and 5** we develop a method for efficient vascularization of kidney organoids through intracoelomic transplantation in chicken embryos. Investigation at a transcriptomic, protein and ultrastructural level demonstrates that a perfused chimeric vascular network is formed in transplanted organoids, consistent with a combination of angiogenesis from host vessels and vasculogenesis from endogenous organoid derived ECs. The vascular network invades the glomerular structures and transplanted organoids display enhanced maturation of glomerular and tubular structures as well as the stromal compartment.

It is reassuring that kidney organoids are vascularized upon transplantation in mice or chicken embryos. It could even be argued that this relieves the need to develop strategies for in vitro vascularization: Upon transplantation of avascular organoids in patients suffering from end stage kidney disease, we might be able to depend on host-derived angiogenesis for vascularization. Indeed, the sparse hiPSC-derived products that have found their way to clinical trials in humans, such as pancreatic islets[12] and heart muscle allografts[13], did not contain a pre-formed vascular network prior to transplantation. However, these products and the vasculature they require are significantly less complex than nephrons, let alone entire kidneys. Although the optimal form and location for future transplantation of hiPSC-derived renal structures are as yet unclear, at least 100.000 functioning nephrons would be required for a clinically relevant effect that could keep a patient off dialysis. If these were delivered to a recipient without a route for blood supply, extensive angiogenesis would be required to assure functional vascularization of the entire graft. In our model of intracoelomic transplantation of bisected organoids containing an estimated 500-1000 nephron-like structures[14], glomerular vascularization commenced 3 days post transplantation, with the blood vessel network further expanding until at least day 7[15]. When transplanting much larger numbers of nephrons, this process is likely to take even longer, leading to a high risk of ischemic injury. In kidney transplantation, increased ischemia time, defined as the time from organ removal from the donor to perfusion with warm blood in the recipient, is a well-known risk factor for delayed graft function, graft failure, and mortality[16-18]. It occurs despite the presence of a fully formed vascular network in the donor kidney which is immediately connected to the host vasculature during transplantation. While cold ischemia time (time in cold storage after removal from the donor and before transplantation in the recipient) will likely be avoided when transplanting hiPSC-derived organoids, a process akin to warm ischemia time (time from organ removal from cold storage to perfusion with warm blood in the recipient) will occur, and be more extensive than the median 35 minutes in donor kidney transplantation[16]. It is therefore highly preferable to equip iPSC-derived kidney tissue with a vascular network to ensure swift perfusion of the entire graft upon transplantation and avoid ischemic injury with loss of functioning nephrons. In addition, functional vascularization of kidney organoids in vitro would expand the possibilities for their application as models of renal physiology and pathophysiology. Microvascular injury plays a role in the most common causes of kidney disease, namely diabetes mellitus and hypertension. Auto-immune diseases such as ANCA vasculitis damage the glomerular endothelium[19], and rare diseases affecting podocytes or the GBM become most apparent in mature glomeruli. None of these conditions can be optimally modelled in an avascular kidney organoid.

Kidney organoid vascularization in vitro

In **Chapter 3**, we present a method for the in vitro co-culture of kidney organoids with 2 microvessels in a microfluidic chip that enables exposure of the ECs to bidirectional flow. The organoids induce angiogenic sprouting from the microvessels, but a connection between organoids and the newly formed vascular network is not established. Causes can be manifold, with a possible role for an inadequate gradient of angiogenic factors, suboptimal EC characteristics, lack of mural cells, and unphysiological flow. Indeed, the application of laminar flow, even when applied over the top of organoids instead of through blood vessels, has been shown to stimulate organoid EC proliferation[20]. Over the past few years, different designs of microfluidic chips have been developed, each containing a chamber for kidney organoids as well as 1 or more lanes lined with ECs that can be exposed to laminar perfusion[21,22]. These set-ups result in sprouting of ECs from the engineered microvessel toward the organoid[21] or from organoid-derived ECs toward the engineered microvessel[22], with the successful establishment of a connection between organoid and microvessel. The direction of sprouting in the model systems seems dependent on the angiogenic gradient, which is influenced by the absence or presence of VEGF as a supplement in the medium. Unfortunately, none of these models succeed in vascularization of organoid glomeruli.

Faced with the challenge of replicating in vitro the extensive vascularization process that occurs in vivo, identifying key changes in EC gene expression profiles upon transplantation can provide important clues. Single cell RNA sequencing (scRNAseq) enables the in-depth analysis of the transcriptome of cell clusters and subclusters within tissues[23]. Despite the availability of several kidney organoid scRNAseq datasets[24-26], the characteristics of organoid ECs have long remained elusive due to their limited number within the organoids. In **Chapter 6**, we deploy the efficiency of our model of intracoelomic transplantation to acquire sufficient human organoid-derived ECs for in-depth scRNAseq analysis. Interestingly, in vitro organoid ECs display little similarity to renal ECs, while transplantation induces the appearance of a subcluster of ECs that resemble fetal kidney arterial / afferent arteriolar ECs. In addition, we identify blood flow and transcription factor Sox7 as likely drivers of arterialization and renal specification.

We did not detect a human EC cluster with a glomerular gene signature in transplanted organoids, despite their presence within podocyte clusters on confocal microscopy imaging. This, in combination with the absence of fenestrae in the glomerular ECs on electron microscopy imaging, suggests that organoid ECs might lack the capacity to fully specialize into glomerular ECs. The differentiation protocol for kidney organoids is focussed on generating nephrons from hiPSCs, with ECs appearing as a byproduct. The culture medium and growth factors are not optimized to support EC maintenance and maturation. Furthermore, even iPSC-derived ECs obtained through dedicated stepwise differentiation protocols have been shown to lack maturity[27,28]. The importance of adequate EC differentiation for interaction with the surrounding renal epithelium is supported by the recent finding that enhanced in vitro vascularization can be achieved by mixing hiPSCs with transgenic hiPSCs that express an inducible ETS translocation variant 2 (ETV2) transcription factor during kidney organoid differentiation[29]. ETV2 induction directly differentiates iPSCs to ECs[29,30]. In these organoids, an EC network is formed that encases podocyte clusters, occasionally invades them, and contains a subcluster of ECs displaying a glomerular gene signature. Of note, the EC-podocyte interaction in this model does not fully resemble the in vivo situation, as the glomeruli have an inside-out appearance with ECs encasing podocyte clusters instead of podocytes surrounding capillary loops. The random mixing of ECs with renal progenitor cells and lack of flow in the system might contribute to this organization.

Conclusion and future perspectives

In the decade that has passed since the publication of the first protocols for the generation of kidney organoids from hiPSCs[31-34], important steps have been taken to improve their suitability for regenerative medicine purposes. Progress has been made with regard to scalability[14,35,36], enhancing morphogenesis[37-39], and generation of immune-evasive iPSCs[40-42]. At the same time, the fast progression in the field of single cell transcriptomics[23] and spatial metabolomics provided new possibilities to characterize kidney organoids. This confirmed a resemblance to first trimester human fetal kidneys[24], but also brought to light variation between organoids from different batches, the presence of off-target cell populations[25,26], and metabolic immaturity of organoid proximal tubules[43]. As our knowledge of kidney organoids expands, we thus become increasingly aware of the remaining challenges.

In focussing on the vascularization of kidney organoids, this thesis addresses one of the major challenges. It provides detailed information on the process of vascularization and maturation of organoids upon transplantation in an efficient and reproducible in vivo model, demonstrates the potential and limitations of endogenous organoid ECs, and identifies modifiable factors for future research. Moving forward, robust vascularization and maturation of kidney organoids in vitro will likely require a source of well differentiated and plastic ECs exposed to laminar flow. Achieving this will advance kidney organoid applicability for modeling renal physiology and disease and be an important step towards suitability for renal replacement therapy.

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