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Kidney organoids: hopes and limitations

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Saragossa (Spain), 3rd October 2020

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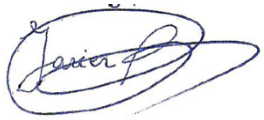

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Abstract

The development of organoids has constituted a major breakthrough in 3D culture technologies. Organoids mimic human organogenesis “on-a-dish”, including early development and adult regenerative processe, and they resemble the original tissue while performing certain tissue functions, thereby organoids open up new avenues to study developmental biology and human pathophysiology. Particularly, patient-derived organoids bear the potential to bring the promise of personalized medicine to reality, since they can be used as patient-specific platforms for drug testing. Consequently, we envisage a scenario whereby disease-specific organoids allow establishing the efficient therapeutic approach and window for that patient. Overall, the combination of the organoid approach with the state-of-art gene editing technology, and the advances in live imaging or biomaterials represent a tour de force that will have a great influence in the close future in our understanding of embryonic development and human diseases, which raises hopes for the development of novel therapies that improve life quality and expectancy.

In particular, kidney research has traditionally been hampered by the lack of suitable *in vitro* models that mimic the high complexity of human kidneys. For this reason, kidney organoids constitute a platform that is already being used to gain understanding on renal pathophysiology and to screen for tubular nephrotoxicity. Although significant advances have been made in kidney organoid generation in the last five years, a number of shortcomings (e.g. vascularization, reproducibility, maturation) have to be overcome to make them suitable for clinical use. The combination of the organoid platform with engineering approaches (e.g. microfluidics, bioprinting and bioreactors) is promising to increase the physiological relevance of organoids. On the other hand, even though kidney organoids may be a game changer to overcome the kidney donor shortage, this approach is not achievable for clinical use in the foreseeable future.

Keywords: organoids, kidney organoids, bioengineering, disease modelling.

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To the memory of the victims of the pandemic

“Cúrame tiempo
Pasa para mí
Y sálvalos a ellos
Sálvalos a ellos.”

Sargento de Hierro, Morgan

“Begin at the beginning,” the King said very gravely,
“and go on till you come to the end: then stop.”

Lewis Carroll, Alice in Wonderland

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1 Kidney Physiology, Development and Regeneration

1.1 Renal Anatomy and Physiology

Human kidneys are complex and highly vascularized organs that play a major role in maintaining normal body functions [1]. Their primary function consists in regulating the fluid homeostasis and the electrolyte and acid-base balances in the organism in order to build up a stable environment for cell metabolism. Consequently, kidneys filter the blood and balance solute and water transport, while excreting metabolic waste products and xenobiotics, and conserving nutrients [1]. Kidneys are also important for controlling blood pressure, synthesis of vitamin D, bone mineralization, and promoting erythrocyte development by producing erythropoietin [2].

The kidney parenchyma is formed by two regions, namely the cortex or outermost and the medulla or innermost (Figure 1A). The functional unit of the kidney is the nephron and they are responsible for most kidney functions [3]. An adult human kidney contains 1 million nephrons on average. The nephron contains more than twenty unique cell types [4]. As shown in Figure 1B, a nephron can be functionally subdivided into a filtration unit, which is known as the renal corpuscle or glomerulus, and a segmented tubular resorption compartment, which can be subdivided into four different segments, namely proximal tubule, loop of Henle, distal tubule and collecting duct (Figure 2) [2].

The glomerulus is a highly specialized capillary tuft located at the proximal part of the nephron and its function consists in filtrating incoming blood by removing excess water and metabolic waste molecules to create pro-urine [5]. The glomerulus contains four resident cell types: glomerular endothelial cells (GECs), podocytes, parietal epithelial cells of Bowman´s capsule, and mesangial cells [2] (Figure 1C). The glomerular filtration barrier (GFB) is made up of an internal layer of GECs that face the capillary lumen, while the podocytes are arranged on the external layer that faces the urinary capsule and both types of cells are kept apart by the glomerular basement membrane [6]. GFB is a size and shape dependent selective molecular sieve that controls the filtering of large molecules while allowing the passage of small molecules and water on the basis of size and charge [6]. In this way, circulating cells (e.g. erythrocytes) and high molecular weight proteins (e.g. albumin) are retained in the vasculature, while water and small molecules (e.g. urea, glucose, amino acids, and ions) are filtrated [2]. These filtrated products pass through the barrier into the glomerular capsule, also known as Bowman´s capsule, and they flow into the renal tubular compartments [6].

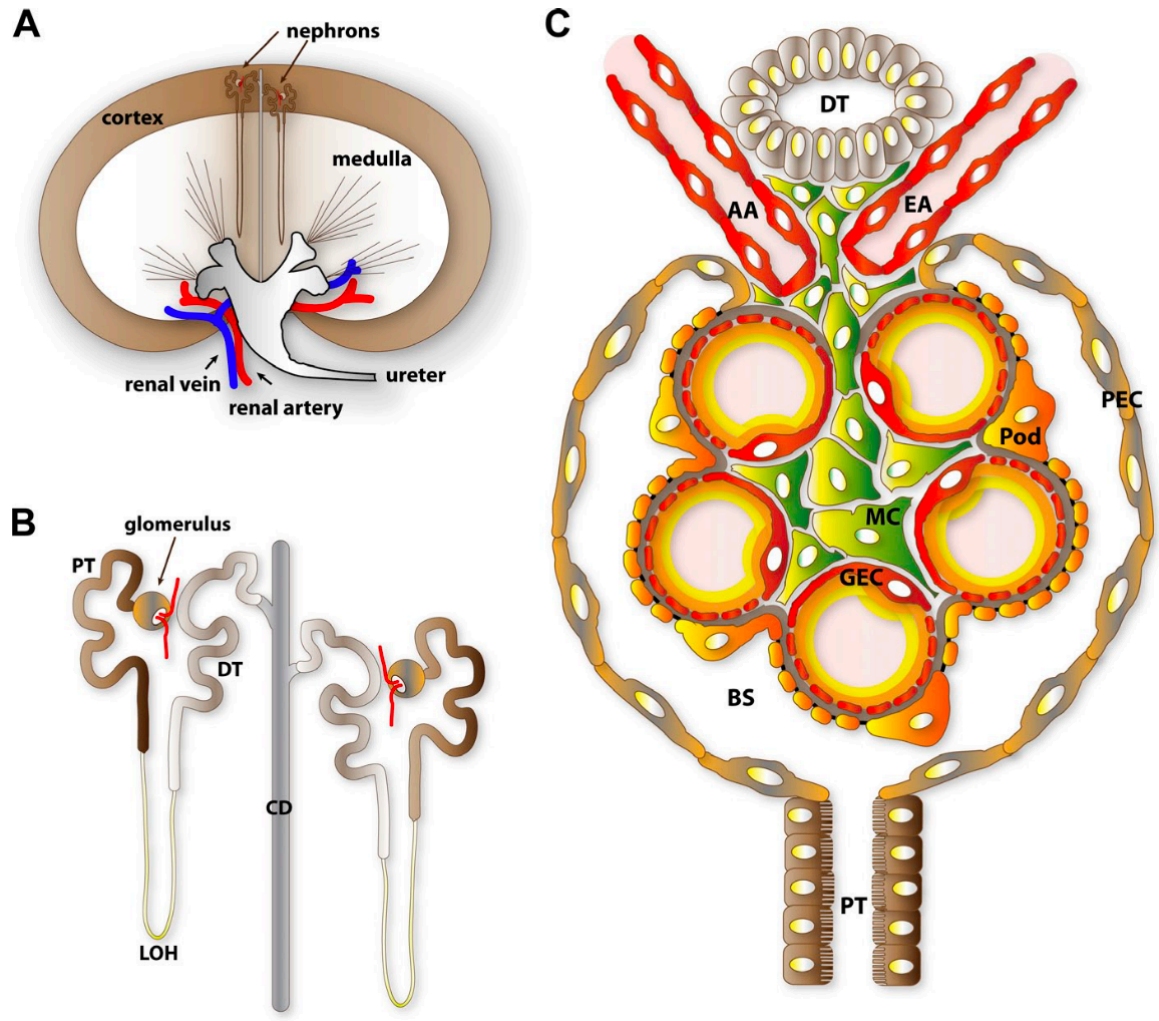

Figure 1: Overview of the kidney anatomy and renal filtration. A) Scheme of the two different regions within kidneys, namely the cortex and the medulla. Glomeruli, which constitute the filtration compartment of nephrons, are located within the kidney cortex. B) Segmental representation of the different sections of nephrons, including proximal tubule, loop of Henle, distal tubule and collecting duct. C) The glomerulus contains four resident cell types: glomerular endothelial cells, podocytes, parietal epithelial cells and mesangial cells. The function of the glomerulus is to serve as size and electric charge-specific barrier to filtrate the incoming blood from the afferent arteriole. GEC stands for glomerular endothelial cell: AA, afferent arteriole; EA: efferent arteriole; Pod; podocyte; MC, mesangial cell; PEC, parietal epithelial cell; PT, proximal tubule; DT, distal tubule; LOH, loop of Henle; CD, collecting duct; BS, Bowman´s space. Adapted from [2].

The first tubular segment is the proximal tubule and it is formed by a leaky epithelium that contains a wide number of specialized transporters that control the reabsorption of different compounds, including electrolytes, bicarbonate and nutrients. Additionally, the secretion of

xenobiotics and toxins also occurs in this segment. The next segment is the loop of Henle that is a long U-shaped tubular section, stretching first down into the renal medulla and then ascending back into the renal cortex. The descending limb of the loop of Henle is mainly involved in water reabsorption, whereas the ascending limb and the following segment, namely the distal tubule, regulate the electrolyte reabsorption. The terminal tubular compartment is the collecting duct and it is composed of two cell types: principal cells and intercalated cells. Principal cells are the most numerous and, under the regulation of aldosterone and vasopressin, they reabsorb water and sodium (in exchange for potassium), thereby contributing to urine concentration. On the other hand, intercalated cells play a major role in the maintenance of acid-base homeostasis by controlling proton and bicarbonate excretion [2], [7], [8]. The collecting ducts eventually converge into a single ureter that acts as one-way outlet for the urine produced in the nephron [9].

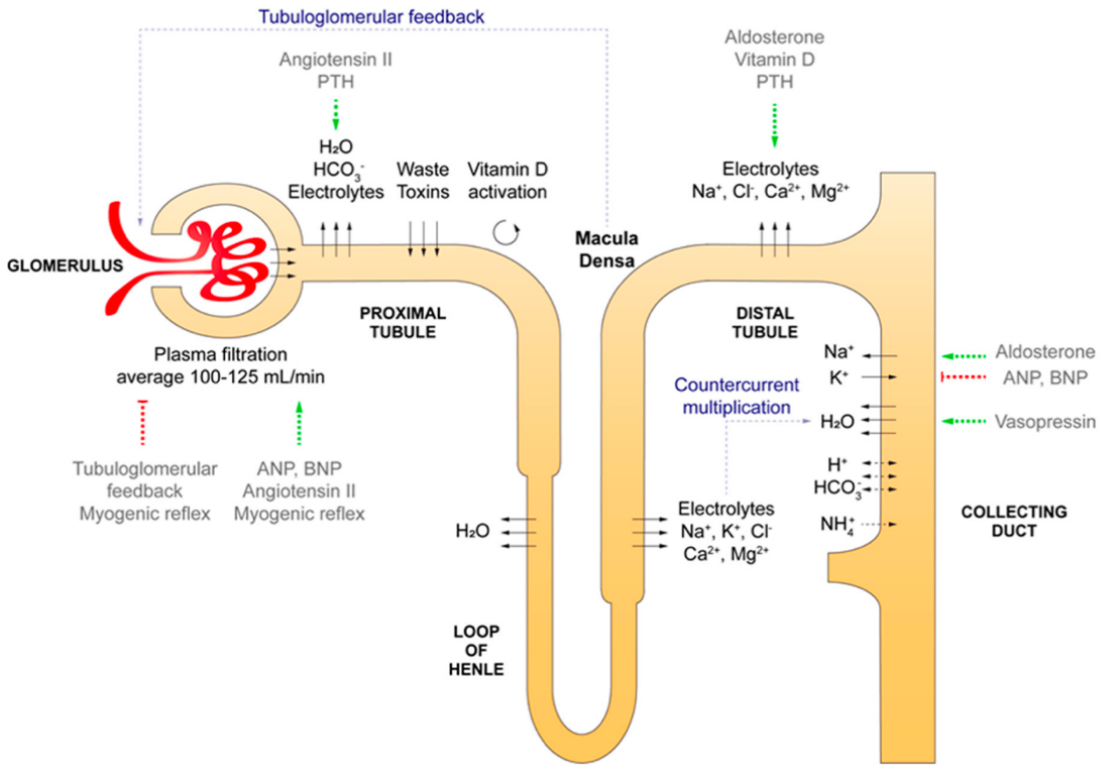

Figure 2: Overview of the main filtration, excretion and endocrine processes that occur at the different segments of the nephron. The nephron is composed of five different segments: glomerulus, proximal tubule, loop of Henle, distal tubule and collecting duct. Important hormones and mechanisms that regulate these processes are highlighted in grey. ANP stands for atrial natriuretic peptide; BNP, brain natriuretic peptide. Adapted from [7].

1.2 Kidney Development

The human kidney represents only one of the three pairs of excretory organs (pronephros, mesonephros, and metanephros) that form during embryogenesis from the intermediate mesoderm (IM) [10]. The pronephros and the mesonephros regress almost entirely during development, whereas the metanephros gives rise eventually to the adult kidney. The embryonic kidney is derived from at least three populations of precursor cells: nephron progenitors form nephrons, ureteric buds give rise to the urinary drainage tract, and stromal progenitors that construct the renal interstitium [9].

Nephron development – termed nephrogenesis – occurs during gestation and it involves different stages that include recurrent epithelial/mesenchyme interactions. The kidney originates from the mesoderm germ layer, specifically from the anterior intermediate mesoderm-derived ureteric bud (UB) and posterior intermediate mesoderm-derived metanephric mesenchyme (MM) precursor tissues. The UB and MM exchange reciprocal chemical signals that regulate, induce, and complete the formation of both structures [11]. The formation of the metanephric kidney is initiated by the ureteric bud, which is an outgrowth from the nephric (Wolffian) duct. The secretion of factors, mainly glial cell line-derived neurotrophic factor (GDNF), by the MM, leads to a sprout of UB towards MM (Figure 3A) [7]. Thereafter, further mesenchymal signals drive ureteric tip proliferation and ureteric tree branching, which eventually will also give rise to the collecting ducts of the mature kidney (Figure 3B, 3C). Simultaneously, the ureteric tip and surrounding stroma secrete factors that regulate both the maintenance of a self-renewing progenitor pool and the commitment into nephron progenitor lineage. In particular, mesenchymal cells surrounding the UB tips condense to become a self-renewing population known as cap mesenchyme (CM), which is characterized by being Sine Oculis Homeobox Homolog 2+ (SIX2+) [12]. Through lineage tracing, it has been demonstrated that SIX2+ CM cells are the nephron progenitor cells (NPCs) that give rise to all the epithelial cell types that are present in the adult nephron [12], [13] (Figure 3D).

Nephron initiation takes place from a WNT4+/SIX2- population located below the ureteric tip, which is known as pretubular aggregate (PA) [11]. In the PA, the activation of LIM homeobox 1 through specific factors (e.g. fibroblast growth factor-8 (FGF8) and Wnt4) leads to a mesenchymal-to-epithelial transition that results in the formation of renal vesicles. These structures initiate a patterning process that polarizes their architecture to generate the S-shaped body. Multiple factors are involved in the formation of the S-shaped body, including Wnt4, FGF and Notch signaling. The secretion of vascular endothelial growth factor (VEGF) by the S-shaped body attracts endothelial cells to the proximal tubule part, which ultimately will be part of the glomerulus. Concurrently, endothelial cells also secrete specific factors (e.g. platelet-derived growth factor) that recruit mesangial progenitor cells that play a major role in

the assembling of the endothelial cells in glomerular capillary loops. The stromal part of the kidney arises from forkhead box D1 (FOXD1+) progenitors that emanate in the periphery of the MM and these progenitor cells give rise to the fibroblasts, smooth muscle, pericytes, and mesangial cells that are present in the adult kidney (Figure 3C and 3D). The elongation and segmentation of the S-shaped body results in more mature nephron-like structures and nephron formation is finally completed around week 36 of gestation [14], [15].

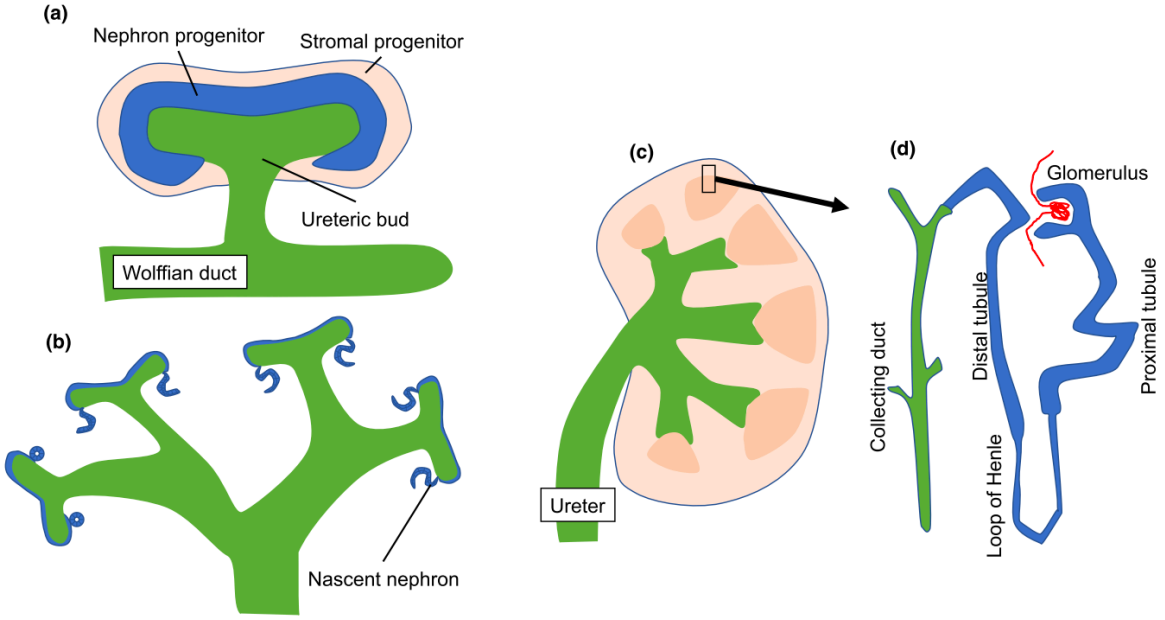

Figure 3: Embryonic development of the human kidney. A) The ureteric bud (green) invaginates from the Wolffian (nephrin) duct into the metanephric mesenchyme, which includes both nephron and stromal progenitors (blue and beige, respectively). **B)** The secretion of specific factors by the metanephric mesenchyme leads to the ureteric tip proliferation and branching and induces nascent nephron progenitor cells to start the differentiation into nephron structures. **C)** Adult kidney anatomy. **D)** Nephron architecture. Ureter (green) and collecting duct are derived from the ureteric bud, whereas glomeruli and tubular structures arise from nephron progenitors (blue). The stromal progenitors give rise to fibroblasts, pericytes and mesangial cells present in the adult nephron (not shown in the Figure). Adapted from [9].

1.3 Kidney Regeneration

Regeneration, broadly defined, stands for the organism intrinsic capacity to recreate lost or damaged tissues, organs or limbs. Neonephrogenesis, which refers to the process of regeneration of every nephron component, is a feature of simple vertebrates, including fish and amphibians [16], [17]. Particularly, elasmobranch fish (e.g. sharks, rays, and skates) have been used as animal models of renal regeneration, since partial nephrectomy induces accelerated nephrogenesis to restore their missing kidney parts [18]. Contrarily, the human kidney responds to partial nephrectomy by stimulating glomerular and tubular hypertrophy, but it is not able to generate new nephrons [16]. To note, in the human kidney, NPCs, which give rise to all the kidney epithelium cells, are completely exhausted shortly after birth [12]. Interestingly, even though the kidney lacks the capacity of the liver to generate a new complete organ after partial resection [19], it maintains the ability to repair and repopulate certain kidney structures, particularly tubular segments, upon acute kidney injury (AKI). Contrarily, the human kidney is characterized by a low turnover rate during homeostasis [20]

This cellular repair process is activated by an ischemic or toxic insult and it is followed by a proliferative burst among proximal tubule epithelial cells, which constitute the main segment of damage following AKI [20], [21]. The origin of these reparative cells has been subject of debate for years [20]–[22]. Initially, it was suggested that these proliferating proximal tubule cells originate by a dedifferentiation process, followed by a proliferative expansion, as shown in Figure 4 [21], [23], [24]. Alternatively, it has also been proposed that proliferative cells may arise from cells outside the kidney, including hematopoietic stem cells or mesenchymal stem cells, or even by adult resident kidney stem cells [16].

To provide insights into the origin of kidney proliferative cells, Humphreys *et al.* [25] crossed the SIX2-GFP-Cre line to a reporter line, which resulted in the expression of the reporter in nearly all kidney epithelial cells, whereas extratubular cells remained unlabeled. Upon ischemic insult, the reporter marker was not diluted, which indicates that proliferative epithelial cells originated from the tubule [25]. However, this result did not exclude the possibility of the commitment of tissue-resident stem cells. To investigate whether an intratubular stem cell population may exist in the proximal tubule, Kusaba *et al.* [24] used an elegant lineage tracing experiment. Their hypothesis was that if a *bona fide* stem cell population resides in the tubular segment, it would not express, by definition of stem cell, a marker of terminally differentiated proximal tubule cells, namely SLC34A1. They found that, upon injury, labelled SLC34A1+ cells started to undergo cell proliferation and, subsequently, to repair the damaged tubular section. Throughout the repair process, labelled cells downregulated the terminally differentiated marker SLC34A1, whereas certain mesenchymal and development markers, such as CD24, CD133, PAX2 and Vimentin were upregulated [24]. Taken together, these results indicate that terminally differentiated proximal tubule cells retain certain plasticity and, upon injury, they

undergo a dedifferentiation and proliferation process to repair the damage. Additionally, Rinkevich *et al.* [26] used long-term lineage tracing to evaluate whether kidney epithelial repair mechanisms are directed through segment-specific cells, or by more promiscuous progenitors. They observed that, upon injury, there is cellular repair in the proximal tubule, loop of Henle, distal tubule and collecting duct in a segment-specific manner, which indicates that their cellular plasticity is restricted to generate a single tubule type [26].

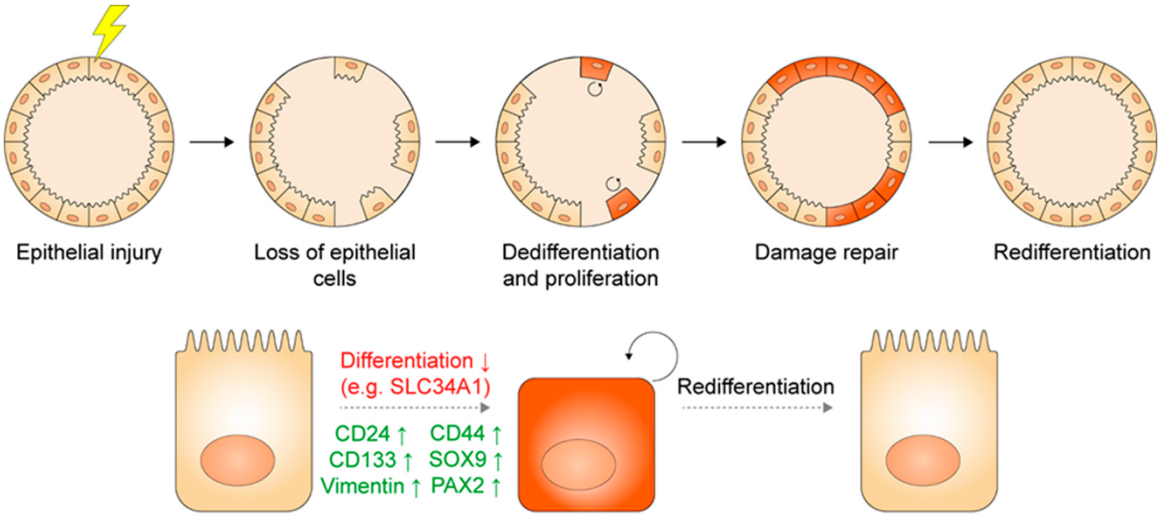

Figure 4: Dedifferentiation and proliferation mechanisms drive the regeneration in the adult proximal tubule. Upon injury and subsequent loss of adult epithelial cells, a dedifferentiation, proliferation and redifferentiation mechanisms are elicited to repair the tubular damage. Throughout this process, differentiated tubular cells lose markers of differentiation (e.g. specific transporters, the brush border), whereas stemness-associated markers are upregulated (e.g. CD24, CD133, Vimentin, CD44, SOX9 and PAX2). Adapted from [7].

Podocytes are a key component of the GFB and podocyte regeneration has also been a significant topic of debate [27]. Adult podocytes, unlike most other renal cell types, are terminally differentiated and quiescent cells, thereby lacking the capacity to proliferate and replace themselves [28]. Podocyte injury and subsequent podocyte depletion have been associated with the development of progressive glomerular diseases [29]. Recently, various studies suggest that human adult podocytes can be replaced to some degree following their loss through transdifferentiation of progenitor cells [30]–[32]. Particularly, a subpopulation of glomerular parietal epithelial cells (PEC) (CD133+/CD24+) have been identified as podocyte progenitors and they display certain ultrastructural features of podocytes [33], [34]. Through lineage tracing, Kaverina *et al.* [35] recently demonstrated that after podocyte loss, PECs migrate towards the glomerular tuft, express podocyte markers and form structures that resemble foot processes. However, as noted by the authors, one limitation of the study, due to the complex transgenic mouse model used, is that it cannot be ruled out that PECs could be aberrantly labelled as podocytes during the experimental procedure [35]. For this reason,

additional innovative approaches would be required to further evaluate podocyte regeneration through PEC transdifferentiation.

1.4 Kidney Diseases

Chronic kidney disease (CKD) refers to an irreversible condition of gradual loss of kidney function that can further progress to end-stage renal disease (ESRD), which is the last stage of CKD. CKD constitutes a major public health problem that affects around 15% of the population nowadays and its prevalence is rapidly increasing worldwide [36]. The rising prevalence of CKD in the elderly is caused by aging and cardiovascular risk factors [37], whereas CKD in the young population is generally a consequence of pediatric tumors, such as Wilms tumor, and congenital abnormalities of the kidney and the urinary tract [38]. As an example of renal pathology, diabetic nephropathy (DN) patients account for an estimated number of about 150 million patients worldwide [39], [40]. DN, also known as diabetic kidney disease, refers to the decline in kidney function observed in both chronic type 1 and type 2 diabetes mellitus patients [9]. The increase in the prevalence of diabetes correlates with a dramatic rise in DN and nowadays this disease constitutes the diabetes-associated complication that causes the highest mortality and morbidity rates and it is the leading cause of CKD and ESRD [41]–[43]. CKD constitutes the long-term diabetes-related complication that has the highest impact on the financial healthcare burden and the quality of life of the patient [44].

Despite the enormous disease burden, the therapeutic armamentarium for CKD remains limited [45] and the development of novel therapeutic approaches is limited by the absence of *in vitro* models that recapitulate the cellular complexity of human kidney [46]. When a patient reaches ESRD stage, the only available therapeutic approaches are either dialysis or kidney transplantation. Given the shortage of kidney donors, the high risk of transplantation and the socioeconomic burden associated with dialysis [47], there is an urgent need of developing advance *in vitro* models that recapitulate kidney complexity to further advance the understanding of renal disease mechanisms and to expedite therapeutic development, but also to generate systems that bear the potential to be used for renal regenerative medicine, particularly for renal replacement therapy.

In the present Thesis, two approaches envisioned to generate kidney tissue that may be used for transplantation, namely interspecies blastocyst complementation (IBC) and kidney organoids, are discussed.

2 Organoids

Biomedical research, and particularly disease modeling, has traditionally relied on using either animal models or two-dimensional (2D) cell cultures on a dish. Cell-based models in 2D, using either primary cultures or immortalized cell lines, are generally used to screen the cellular effects of drug efficacy or cell cytotoxicity [48]. Nonetheless, these cells do not possess a complex extracellular cell matrix (ECM) and they do not establish physiological cell-cell and cell-matrix interactions that are required to mimic the organ function and disease state as present *in vivo* [6], [49]. Moreover, 2D-cultured cells suffer from metabolic changes and dedifferentiation, thereby providing limited translational outcomes [50]. Model organisms and, particularly, mice models have generally been used to understand human development and to elucidate the underlying mechanisms of human diseases; however, animal experiments are generally expensive and time-consuming and they are not suitable to monitor biological changes in real-time [6], [51]. Furthermore, these models have limitations when differences between humans and animals emerge [52]. Over a decade ago, the discovery of induced-pluripotent stem cells (iPSCs) by Takahashi and Yamanaka supposed a major finding for the development of new human disease models and platforms for drug screening [53]. The recent improvement in 3-dimensional (3D) culture techniques, particularly the development of organoids, has made possible the advent of new research tools that have great potential applicability for disease modelling and translational research (Figure 5) [54].

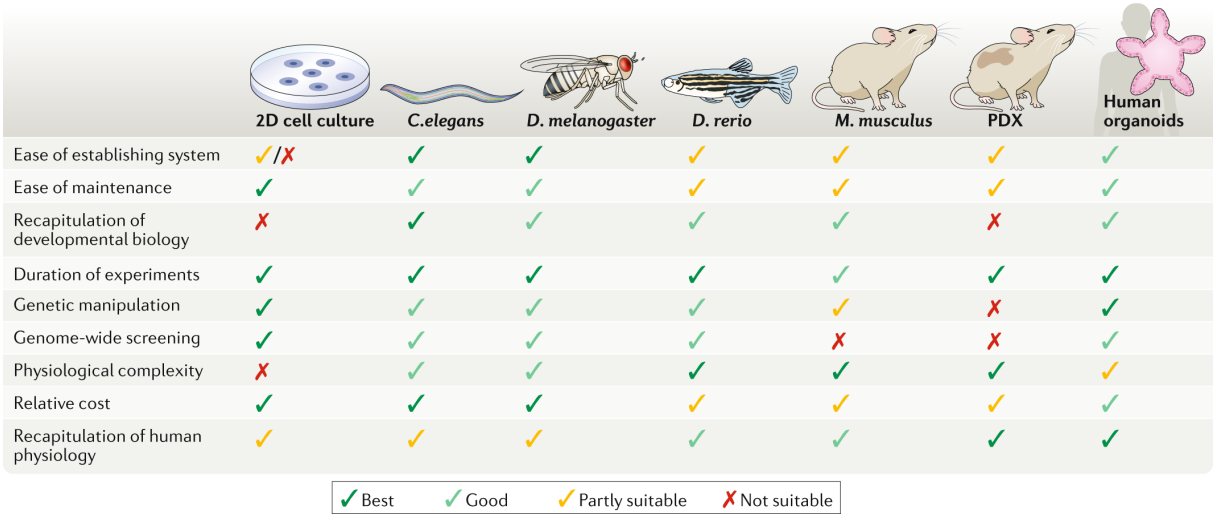

Figure 5: Overview of the advantages of organoids in comparison to other model systems. The most habitual model organisms in biomedical research are *Caenorhabditis elegans*, *Drosophila melanogaster*, *Dario rerio* and *Mus musculus*. These model organisms, along with 2D cell culture and patient-derived xenografts (PDX), have traditionally been the platforms used for biological studies. The relative benefits and limitations of human organoids in comparison to these platforms are outlined. Relative scores are illustrated as being the best (dark green tick), good (light green tick), partly suitable (yellow tick) and not suitable (red cross). Adapted from [55].

Organoids constitute a 3D assembly of cells, typically from a human source, which can include differentiated cells, stem cells or both, that withhold the potency of self-organization and display an organ-like behavior [56]. In particular, self-assembly and differentiation are hallmark features of organoid formation and the signaling cues that regulate these processes are provided by the ECM, the growth factors in the medium and the cell types that conform the organoid themselves [57]. Organoids can be derived from either tissue-specific adult stem cells (ASCs) or from pluripotent stem cells (PSCs), including both embryonic stem cells (ESCs) and iPSCs [58]. These two distinct organoid systems possess unique and complementary features, since PSC-derived organoids mimic organogenesis during embryonic development and usually resemble fetal-stage tissues, whereas ASC-derived organoids recapitulate adult tissue biology [54], [59].

Their 3D architecture represents a more near-physiological condition compared to 2D culture, which facilitates obtaining accurate model conditions to study organ development, disease progression or *in vitro* drug screening (Figure 6) [58], [60]. In the long-term, organoids and their ability to recreate organ development on-a-dish have been conceived for therapies involving transplantation of organoid-based solid organs, thereby representing a promising approach to overcome the shortage of organ donors [57].

In 2009, Clevers and co-workers first reported that intestinal organoids can be established from Lgr5 (leucine-rich repeat-containing G protein-coupled receptor 5)-positive stem cells by imitating the *in vivo* stem cell niche environment [61]. To date, ASC-derived organoids from all the principal organs have been established, including intestine [61], liver [62], colon [63], pancreas [64] and stomach [65]. These ASC-organoids are generally established by dissociation of the primary tissue, sorting tissue-specific ASCs, and culture of the stem cell population using Wnt activators [58], [66].

On the other hand, PSCs are defined by an unlimited capacity for self-renewal and the ability to differentiate into the three primary germ layers, namely endoderm, mesoderm and ectoderm [67]. PSC-derived organoids are generated through directed differentiation by mimicking the specific combinations of growth factors (e.g. fibroblast growth factor (FGF), retinoic acid (RA) or transforming growth factor beta (TGFβ)) that drive the germ layer induction and the organ generation during embryonic development [3]. The generation of organoids from PSCs was pioneered by Sakai and colleagues by generating self-assembled structures that resemble the cerebral cortex [68] and optic cup [69] using ESCs as the cell source. Hitherto, PSCs have been successfully differentiated into organoids of a number of organs, including the brain [70], kidney [71], [72], pancreas [73], intestine [74] or liver [75]. Notably, Hofbauer *et al.* [76] recently unveiled the generation of PSC-derived cardiac organoids, termed cardioids, being the heart the last missing organ to be generated with organoids.

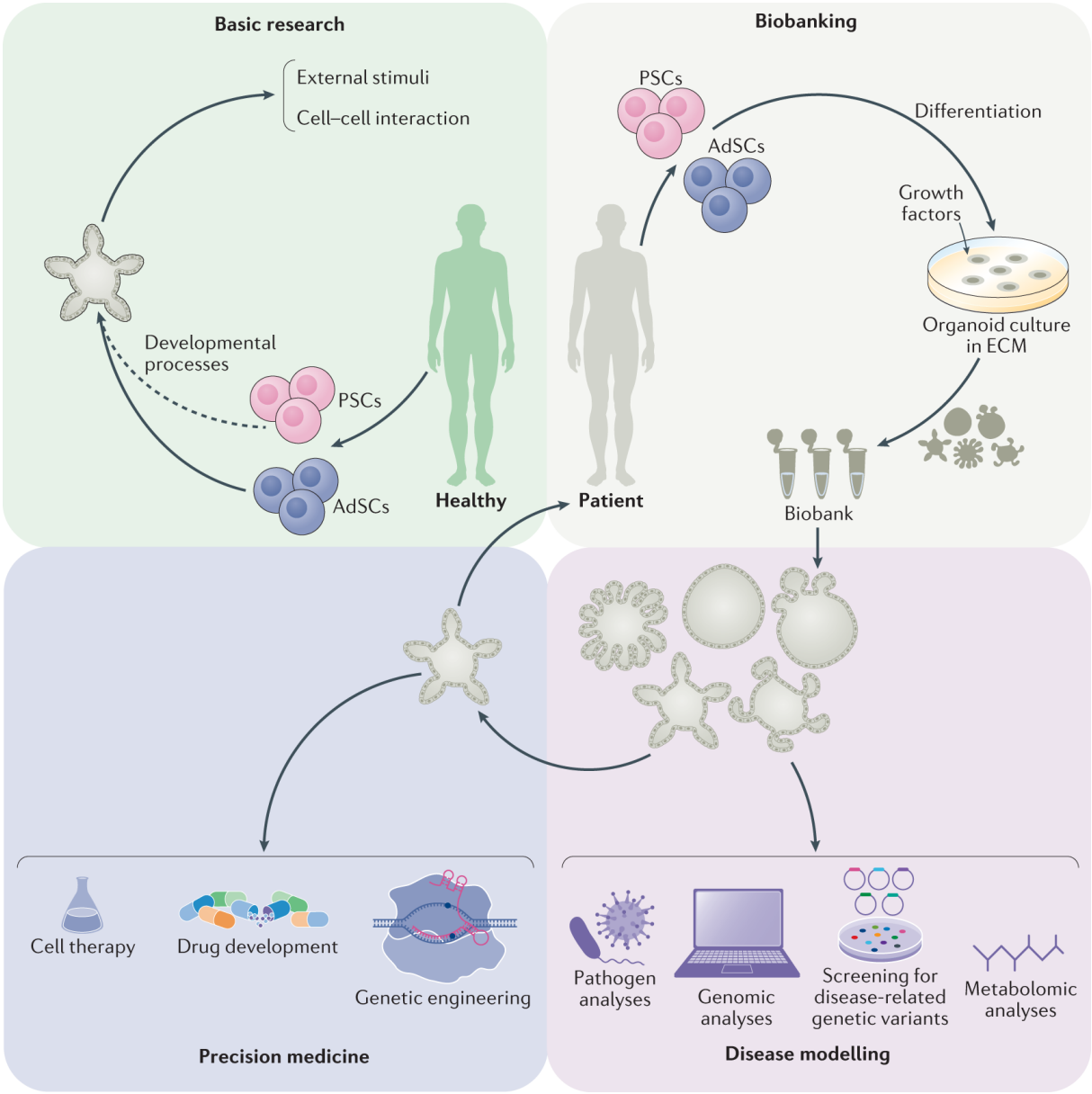

Figure 6: Translational applicability of organoids. Organoids can be used for (1) model systems for basic research, including human biology research, aiming to understand human development and organogenesis processes; (2) biobanking, whereby patient-derived organoids are obtained and stored and can be used for future research purposes; (3) disease modelling, to unveil the mechanisms that regulate and drive disease progression of various human pathologies (e.g. infectious diseases, inheritable genetic disorders or cancer); (4) precision medicine, in which patient-derived organoids can be screened to predict drug response and they can also be derived for regenerative medicine purposes. Adapted from [55].

3 Kidney Organoids

The generation of kidney organoids, which are made up of nephron-like structures that are composed of early glomeruli clusters that are connected to tubular structures, and resemble kidney functions, has supposed a major breakthrough within the nephrology field [77], [78]. Kidney organoids are regarded as an important potential platform to study human kidney development and to model kidney diseases that affect glomeruli and renal tubules, to perform *in vitro* drug screening to test nephrotoxicity and, ultimately, for regenerative therapies [79]. In particular, kidney organoids represent a promising tool for *in vitro* renal disease modelling, since they can be combined with systematic analysis using state-of-the-art genome editing tools, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems 9 (Cas9) to perform drug screens to identify novel therapeutic targets [18].

The development of kidney organoids represents a promising tool to overcome the limitations of 2D culture models or animal models. In particular, animal models have contributed and will continue being of exceptional applicability to gain insights into disease modelling [80]. Mouse models resemble the cellular complexity of human kidney, but they are limited in mimicking the pathophysiological progression of human diseases. Subsequently, as previously indicated, the historical reliance on animal models has sometimes been limited by their translational applicability and has supposed a challenge to address questions that are specific to human biology and human diseases [50], [55]. For example, a major barrier to study polycystic kidney disease (PKD) has been the lack of an animal model that faithfully recapitulates PKD-specific cytogenesis from distal nephrons, thereby the combination of genome editing technologies and kidney organoids derived from human pluripotent stem cells (hPSCs) could result in a much more accurate model for renal diseases with a genetic basis [80], [81]. Moreover, in comparison to animal models, organoids bypass their ethical concern and they also represent a much more flexible and defined-component system that allows continuous tracking for longer periods of time [81].

3.1 Protocols to Generate Kidney Organoids

Hitherto, two different approaches have been established to produce kidney organoids using pluripotent stem cells (PSCs) as a cell source: directed differentiation and direct reprogramming. Directed differentiation protocols rely on multi-step procedures involving the usage of numerous growth factors and chemical compounds to produce nephron-like structures [71], [82], [83]. The protocols to generate kidney organoids are based on mimicking the environmental cues, including both physical and chemical ones, that are present in the development of the kidney in an embryo [79], [84]. On the other hand, direct reprogramming protocols (e.g. Hiratsuka [85], Vanslambrouck [86], Kaminski [87], Hendry [88] and Papadimou [89]) use exogenous transcription factor (TF) expression vectors to induce the differentiation of hPSCs into kidney lineage. Direct reprogramming of differentiated cells into a different organ-specific cell type has been feasible by using defined transcription factors, thereby bypassing their pluripotency state [87]. For example, fibroblasts have been directly reprogrammed so far to generate a variety of cell types (e.g. neurons [90], cardiomyocytes [91] or hepatocytes [92]).

The protocol established by Hiratsuka *et al.* [85] is an example of direct reprogramming approach to generate kidney organoids. This protocol is based on the high efficiency transfection (>90%) of synthetic messenger RNAs (mRNAs) [93], thereby bypassing the use of viral and plasmid expression vectors, which possess limitations in clinical applications [87], [88]. In this way, two different sets of TFs were identified to generate kidney tissues using hPSCs as a cell source. The first set of TFs (FIGLA, PITX2, ASCL1 and TFAP2C) achieves the induction of NPCs from hPSCs, whereas the second set (HNF1A, GATA3, GATA1 and EMX2) is used to complete the induction into nephron epithelial cells [85]. The sequential administration of these TFs and the subsequent 3D suspension culture achieve, in 14 days, kidney tissues, which display the features of proximal and distal renal tubules, and glomeruli [85]. Consequently, direct reprogramming represents an alternative approach to generate nephron-like structures *in vitro*. However, one defect of reprogramming approach is that cells derived via direct conversion less closely resemble their native counterparts than those generated by directed differentiation [94]. For this reason, directed differentiation is the most promising approach to recreate organs for disease modelling and regenerative medicine.

Over the past 5 years, a number of directed differentiation-based protocols (e.g. Morizane [71], Takasato [72], Freedman [82], Taguchi [83] and Garreta [95]) have been developed to generate 3D kidney organoids from either using iPSCs or ESCs as a cell source and they differ in terms of length of culture and growth factors used. These protocols for ESC- and iPSC-derived kidney organoids yield nephron structures, including glomerular, proximal tubular, and distal tubular, which are generally surrounded by stromal cells and endothelial cells (ECs) [71], [72], [96].

Initial studies to derive kidney tissue from hPSCs focused on the identification of growth factors (e.g. bone morphogenic proteins (BMP4, BMP7), retinoic acid, hepatocyte growth factor (HGF), and insulin-like growth factors that induce the differentiation into kidney lineage [97]–[99]. From that basis, differentiation protocols to generate kidney organoids can be subdivided into two steps: i) directed differentiation into the IM and NPCs and ii) differentiation of NPCs into nephron structures. Additionally, since these protocols were designed to follow the steps of kidney embryonic development *in vivo*, intermediate different populations of each step of differentiation (e.g. late mid-primitive streak, posterior IM, NPCs, pretubular aggregates, renal vesicles, and, ultimately, nephrons) can also be induced [71].

The protocol developed by Taguchi and Nishinakamura [83] contains both UB- and MM-derived structures. To achieve the generation of 3D kidney organoids containing both UB and MM-derived compartments, each compartment is initially generated separately and then they are subsequently co-cultured, which results in organized nephron structures that include a CD system. Initially, the primitive streak is induced using Activin A and BMP4 treatment, followed by BMP4 and CHIR99021. Thereafter, treatment with RA, FGF9, SB431542 (TGF inhibitor), and LDN193189 (BMP inhibitor) are used to induce anterior IM formation. Subsequently, the generation of Wolffian duct was triggered using RA, CHIR99021, FGF9, and LDN193189 and sorting for CXCR4+/KIT+ cells. Lastly, UB is induced using a treatment that includes Rho-associated kinase inhibitor (Y27632), RA, CHIR99021, FGF9, FGF1, LDN193189, and GDNF. On the other hand, to generate the MM, the IM was generated following a sequential treatment using i) Activin A, ii) CHIR99021, iii) a combined treatment of Activin A, CHIR99021, BMP4 and RA. Thereafter, the MM lineage was directed using FGF9 and low CHIR99021. The complexity of the Taguchi protocol remains as a major drawback for the generation of kidney organoids for high-throughput studies.

On the other hand, the protocol established by Takasato and Little [72] simultaneously differentiates anterior and posterior IM in order to generate epithelial derivates from both the MM and UB. In the Takasato protocol (Figure 7), hPSCs are initially treated with the glycogen synthase kinase inhibitor, CHIR99021, thereby activating WNT signaling to trigger first the differentiation into the primitive streak, and subsequently, into anterior and posterior IM. A short or longer treatment with CHIR99021 favors the predominant induction of anterior or posterior IM, respectively. Consequently, the length of the CHIR99021 treatment determines the ratio of collecting ducts and nephron segments in the organoid. Thereafter, progenitor cells are dissociated and established as a kidney progenitor aggregate to generate kidney organoids. To maximize nephron formation, the organoids are stimulated with a short pulse of CHIR99021 followed by FGF9 treatment.

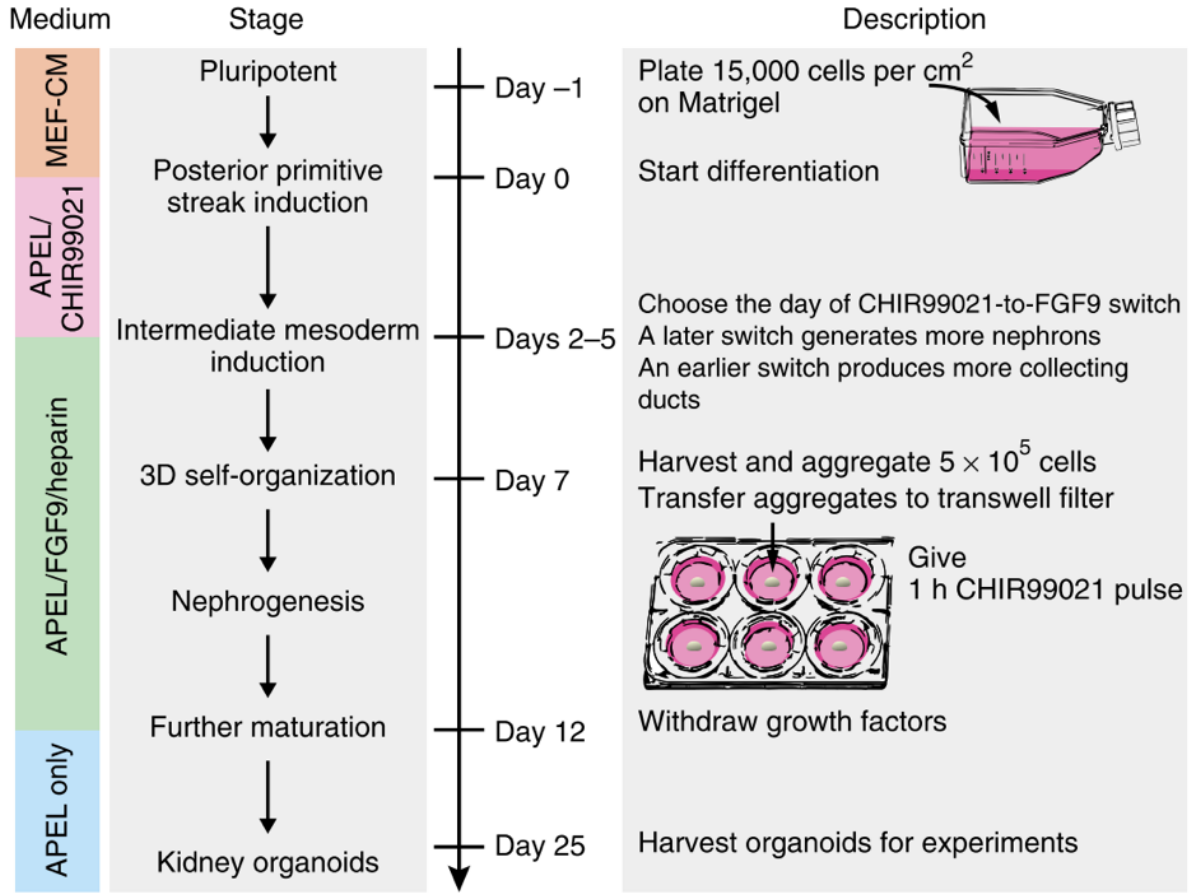

Figure 7: Schematic timeline of the Takasato protocol to generate kidney organoids. The protocol is based on directed differentiation of human pluripotent stem cells (hPSCs) into renal lineage using sequential change of culture media and specific growth factors. Initially, induction into intermediate mesoderm is triggered using APEL medium supplemented with 8 µM CHIR99021. Thereafter, nephron lineage induction is achieved using APEL medium supplemented with 200 ng/mL FGF9 and 1 µg/mL heparin. Finally, all growth factors are withdrawn in the last step. FGF9 stands for fibroblast growth factor 9; MEF, mouse embryonic fibroblast. Adapted from [72].

Finally, the protocol developed by Morizane *et al.* [71] (Figure 8) recapitulates metanephric kidney development *in vitro* to achieve NPC generation with a 80-90% within 9 days of culture (Figure 9A). First, they induce the primitive streak, which is the progenitor population for both endoderm and mesoderm, with CHIR99021, which is followed by a treatment using Activin A to induce the posterior IM. Thereafter, FGF9 treatment achieves the differentiation SIX2+ NPCs. NPCs are subsequently dissociated and transferred as a cell aggregate into low-adherent 96-well plates and a transient CHIR99021 pulse followed by FGF9 treatment leads to the generation of renal vesicles that self-pattern into nephron-like structures, including podocytes, proximal tubule, loop of Henle and distal tubules, and their 3D organization resembles the nephron *in vivo* (Figures 9B, 9C and 9D) [100]. However, since differentiation

into NPC lineage occurs prior to self-organization, kidney organoids developed using the Morizane protocol lack CD structures.

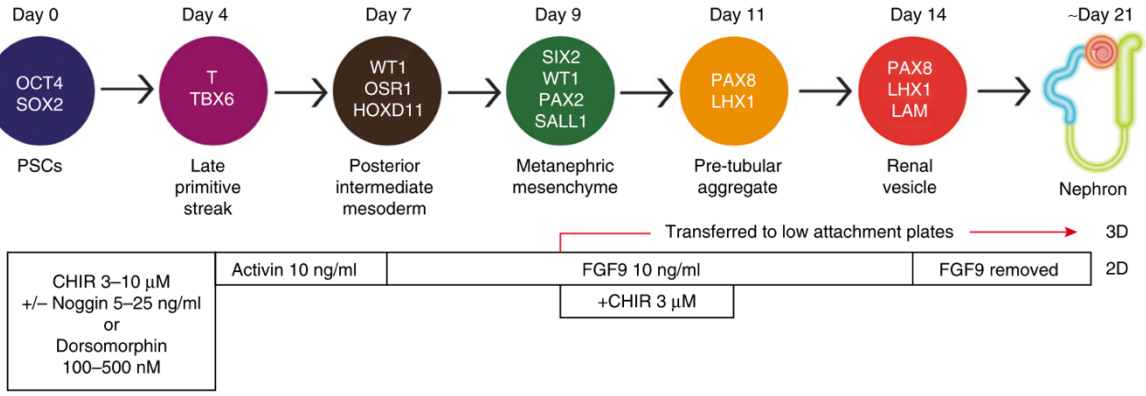

Figure 8: Overview of the Morizane protocol to produce kidney organoids from human pluripotent stem cells. The diagram displays the different stages of the differentiation protocol in a timely manner, including the markers used for quality control for each step. The growth factors and small molecules used in each stage, including their concentration, are also shown. PSC stands for pluripotent stem cell; CHIR, GSK-3ß inhibitor CHIR99201; FGF, fibroblast growth factor. Adapted from [71].

Overall, despite the differences in terms of culture conditions and growth factors between the above described established protocols to generate kidney organoids, the resulting organoids display many similarities. Remarkably, in all cases, kidney organoids contain podocytes (WT1+/PODXL+), proximal tubules (LTL+/CUBN+), and distal tubules (ECAD+/CDH1+), as demonstrated using immunostainings and confocal microscopy [71], [72], [83]. The presence of ECs (CD31+/vWF+) [72], [82], mesangial cells [72] and ascending loop of Henle (UMOD+) [71], [72] are described only in some protocols. Remarkably, kidney organoids established using the Morizane and the Takasato protocol have demonstrated certain renal tubule functions, including sensitivity to nephrotoxicity substances (e.g. cisplatin) and dextran absorptive capacity [71], [72]. Additionally, Wu *et al.* [101] used single cell RNA-sequencing to compare the resulting kidney organoids generated using the Morizane [71] and the Takasato [72] protocols. This study demonstrated that both resulting organoids were similar in terms of the cell populations present, but slight differences in the percentages of each population were also observable [101].

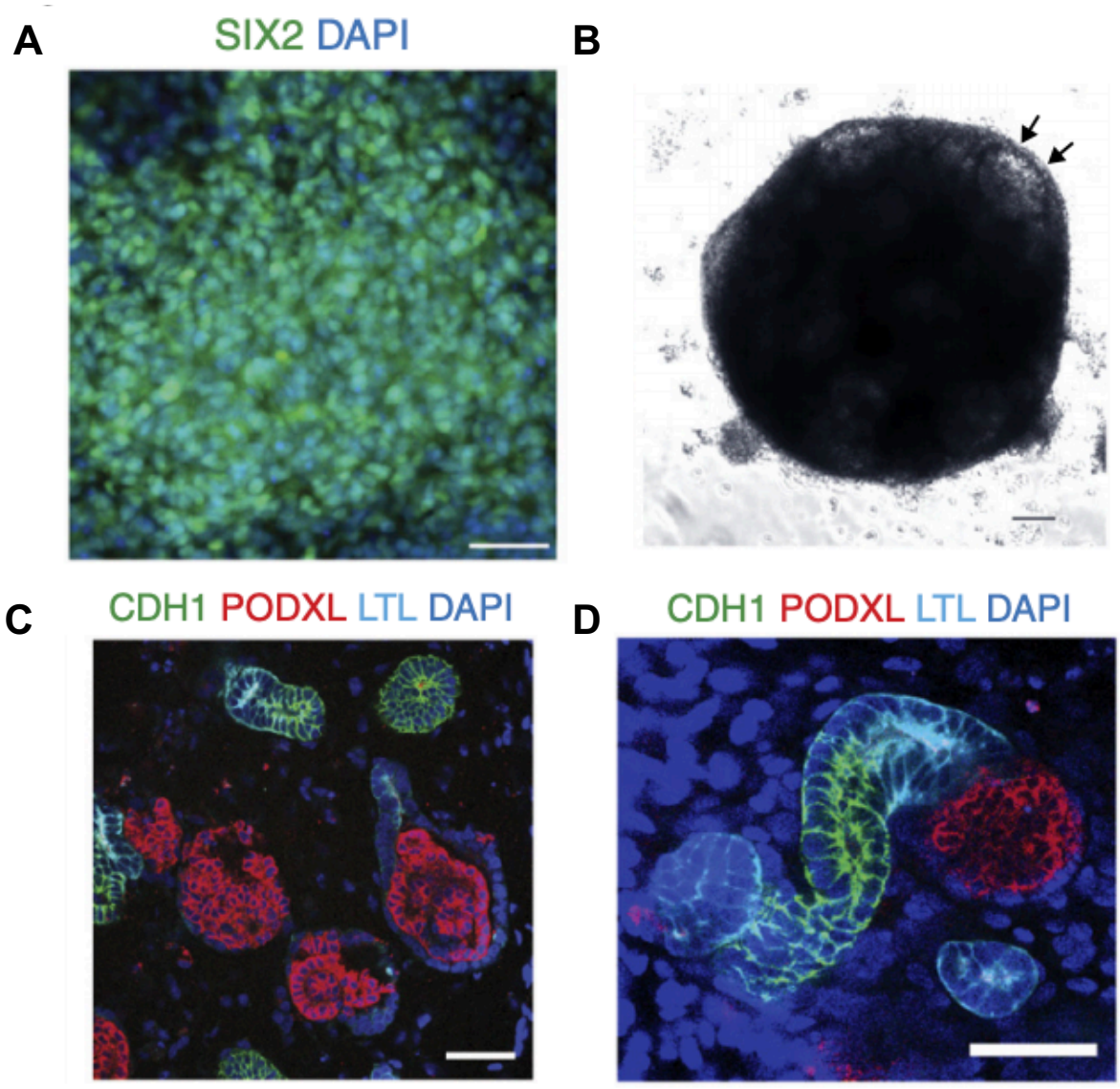

Figure 9: Immunostaining and morphological appearance of nephron progenitor cells (NPCs) and kidney organoids generated following the Morizane protocol. A) Immunocytochemistry for SIX2, marker of NPCs, at day 8 of differentiation. Scale bar, 50 µm. **B)** Representative bright-field imaging of a 3D kidney organoid on day 21. Arrows point glomerular structures. Scale bar, 100 µm. **C)** Immunohistochemistry of frozen sections of 3D kidney organoids at day 21 of differentiation to identify nephron segments. Scale bar, 50 µm. **D)** Whole-mount immunostaining of 3D kidney organoids at day 28 of differentiation. Scale bar, 100 µm. CDH1 stands for cadherin 1 (also known as E-cadherin; a loop of Henle and distal tubule marker); DAPI, 4′,6-diamidino-2-phenylindole; LTL, *Lotus tetragonolobus lectin* (a proximal tubule marker); PODXL, podocalyxin (a podocyte marker); SIX2, Sine Oculis Homeobox Homolog 2. Adapted from [71].

3.2 Applications of Kidney Organoids

Kidney organoids have already been used to study kidney development during embryogenesis [96], [100], to model kidney diseases [102], [103] or to evaluate tubular nephrotoxicity of different compounds [82], [100]. Additionally, kidney organoids also bear the potential to be used in regenerative medicine for renal replacement therapy [80].

In the present section, three current applications of kidney organoids will be highlighted: disease modelling of genetic renal diseases with particular focus on PKD modelling, the use of kidney organoids to model viral infections and the establishment of patient-derived kidney tumoroids to study cancer progression and evaluate drug efficacy in a patient-specific manner.

3.2.1 Disease Modelling of Genetic Kidney Diseases

A particular promising application of kidney organoids is to model genetic renal diseases (e.g. PKD, Alport syndrome, medullary cystic kidney disease) [104] to gain understanding of the pathophysiological mechanisms, to discover therapeutic targets and for drug screening. For that purpose, kidney organoids can be established using iPSCs from accessible somatic cells (e.g. skin fibroblasts or leukocytes) from the patient suffering from the renal genetic disorder [82]. Alternatively, due to the high variability between iPSC cell lines in generating kidney organoids, it is generally preferable to use gene-editing tools in iPSC lines to generate mutant kidney organoids that mimic genetic renal diseases, while maintaining an isogenic non-mutated control [72], [79].

Kidney organoids have been extensively used to study PKD [82], [105]. In particular, autosomal dominant PKD (ADPKD) constitutes the most common kidney genetic disorder and it is the cause of approximately 10% of the patients with ESRD. Phenotypically, ADPKD is characterized by the emanation from the renal epithelial cells of fluid-filled sacs, namely cysts, that grow over time and lead to kidney enlargement and renal function decline that eventually derives into CKD in approximately 50% of the patients [106]. ADPKD is associated with heterozygous mutations in the *PKD1* and *PKD2* genes, which encode for the renal ciliary proteins polycystin-1 and polycystin-2, respectively [107]. However, the limited knowledge of the function of the PKD-associated proteins has hampered the understanding of the disease progression as well as the development of new therapies.

Hitherto, PKD disease modelling has mainly relied on mouse models or primary renal cells isolated from ADPKD patients [105]. Particularly, *PKD1* or *PKD2* knockout mice are incompatible with long survival times, whereas *PKD1* or *PKD2* heterozygotes display a very

mild cystic disease, thereby they are not suitable to mimic human ADPKD disease progression [108]. For this reason, PKD-mutant organoids represent an alternative platform for disease modelling of the pathology.

Recently, Freedman *et al.* [82] engineered PSC-derived kidney organoids to model ADPKD. For that purpose, they initially used CRISPR-Cas9 genome editing for hPCSs that harbor mutations in either PKD1 or PKD2 and, subsequently, mutated-hPSCs were differentiated into kidney organoids. Initially, they established PKD-mutated organoids using low attachment plates, which resulted in the formation of large cysts that phenocopy ADPKD, as shown in Figure 10. Additionally, PKD organoids can also be used to unravel the molecular mechanisms that drive the cystogenesis. Particularly, Cruz *et al.* [105] investigated the role of the ECM microenvironment in the development of cysts. Embedding of *PKD1*-mutant organoids into collagen droplets diminished cyst formation, whereas the removal of the adherent cues of the collagen hydrogel promoted the formation of cysts, which indicates the primary role of the surrounding ECM and the adhesion for the early stages of the disease [105].

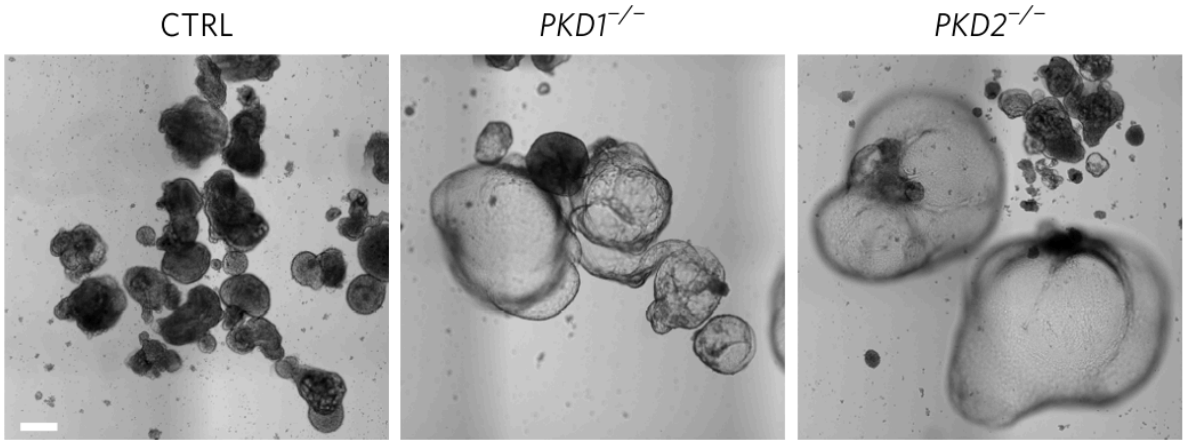

Figure 10: PKD-mutant kidney organoids are an efficient model of polycystic kidney disease cystogenesis. Using CRISPR-Cas9 genome editing, human pluripotent stem cells (hPSCs) harboring loss of function mutations in either *PKD1* or *PKD2* are generated and mutated hPSCs are used to establish kidney organoids. *PKD1* and *PKD2* knockout kidney organoids result in the formation of the characteristic cysts of PKD. PKD stands for polycystic kidney disease. Adapted from [103].

Collectively, disease modelling of PKD using kidney organoids serves as a proof-of-concept of the potential of the organoid platform to gain understanding in the (patho)physiological process of the disease and to discover new therapeutic approaches for the treatment of hereditary kidney diseases.

3.2.2 Kidney Organoids for COVID-19 Research

The understanding of the viral biology has traditionally relied on *in vitro* models, generally cell lines of monkey or human origin, that permit viral replication. During the Zika virus (ZIKV) epidemic in 2015, organoids already proved their applicability to understand the infective and pathogenetic mechanisms of virus [109]. In particular, it was observed that there was an association between ZIKV infection and the development of congenital abnormalities, including microcephaly [110]. Using brain organoids, it was demonstrated that ZIKV is able to cross the placenta and preferentially infects neural cell precursors, which subsequently leads to the development of microcephaly [109].

In early December 2019, an outbreak of acute respiratory illness caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was detected in Wuhan, China [111]. SARS-CoV-2 causes coronavirus disease-19 (COVID-19) and this respiratory illness has spread rapidly by human-to-human transmission, leading to outbreaks worldwide, and it has caused substantial mortality and morbidity [112]. On March 11, 2020, COVID-19 was declared by WHO as public health emergency of pandemic proportion. The disease generally courses with mild influenza-like symptoms, including cough, fever, and fatigue; however, for a minority of patients, the disease worsens and it results in complications (e.g. acute respiratory distress syndrome and multi-organ failure) and eventually to death, particularly in older patients that present comorbidities [112], [113].

The virus utilizes the host angiotensin-converting enzyme 2 (ACE2) as a key receptor to infect human cells. To understand the infective mechanisms of SARS-CoV-2, multiple research groups have used organoid approaches. Particularly, to evaluate the suitability of kidney organoids to study the tissue tropism of SARS-CoV-2, the expression of the ACE2 receptor in adult kidneys and kidney organoids was examined using sc-RNA-seq datasets. Interestingly, as shown in Figure 11, ACE2 expression is restricted to proximal tubule cells in both healthy adult kidneys and kidney organoids, which proofs the applicability of kidney organoids as *in vitro* platform to study kidney infection by SARS-CoV-2. In this way, Penninger and colleagues [114] showed that SARS-CoV-2 is able to infect *in vitro* capillary and kidney organoids. They also demonstrated that human recombinant soluble ACE2 inhibits the infection of SARS-CoV-2 on organoids [114], which represents a promising approach for the disease since, to date, there are no clinically approved specific countermeasures for COVID-19. Similarly, ASC-derived organoids, which also display ACE2 receptor on the apical membrane, have also been used as *in vitro* models for SARS-CoV-2 infection [115], [116]. Intestinal organoids served to identify the host-cell membrane-bound serine proteases TMPRSS2 and TMPRSS4 that play a major role in the cleave of SARS-CoV-2 spike protein, thereby facilitating viral entry into the cell [116].

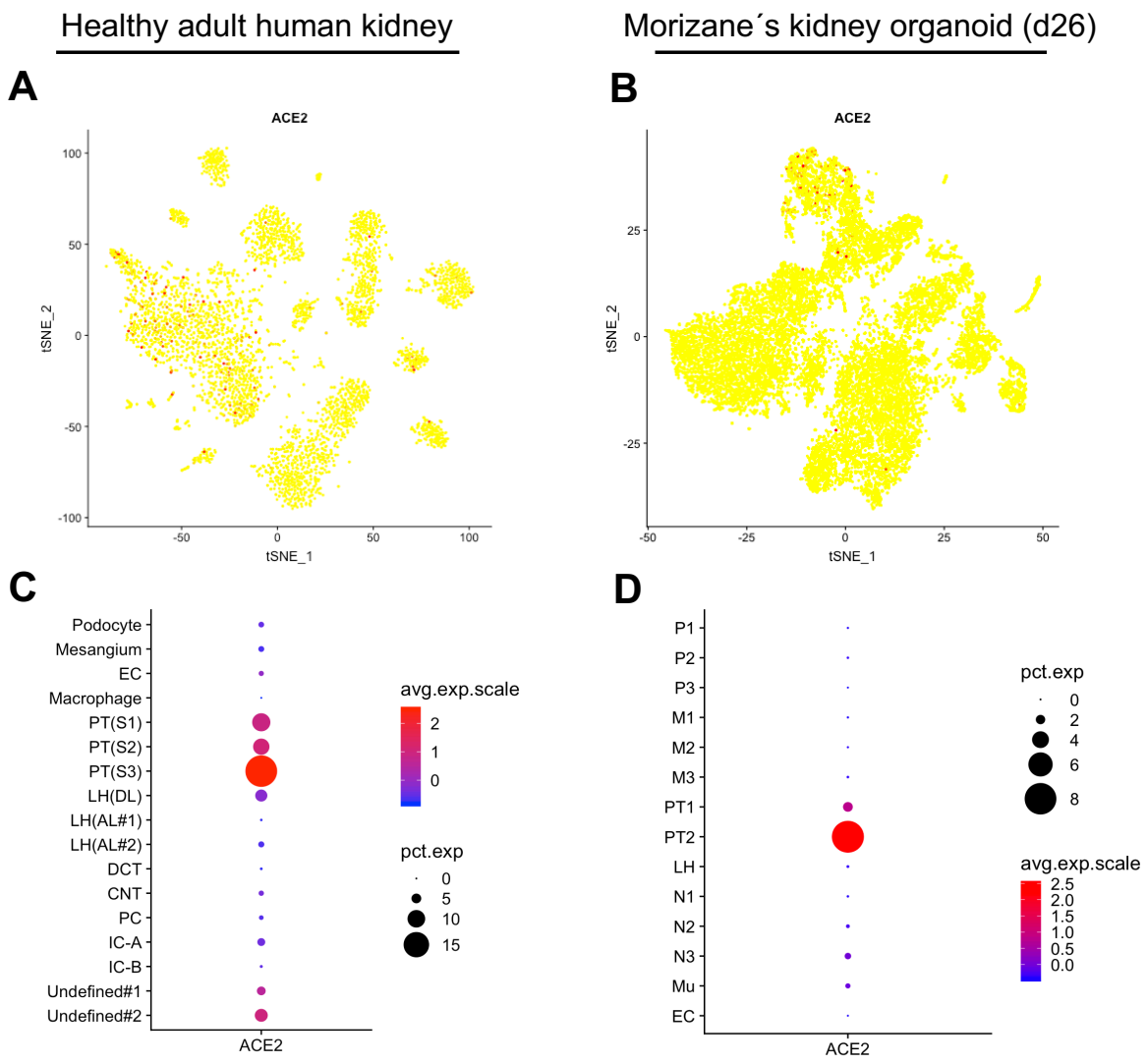

Figure 11: ACE2 is expressed in human kidney organoids. A), B) t-SNE analysis of single-cell RNA-sequencing of healthy adult kidney **(A)** and human kidney organoids (day 26) established using the Morizane protocol **(B)**. ACE2 expression in each cell cluster is highlighted in red. **C)** ACE2 expression in healthy adult kidney is mostly localized in proximal tubule cells. **D)** ACE2 expression in kidney organoids is restricted to PT2 cluster. EC, endothelial cell; PT; proximal tubule; LH, loop of Henle; PC, podocyte; IC, intercalated cell; P, podocytes; M, mesenchyme; N: neuron; Mu, melanocyte. Adapted from [117].

3.2.3 Kidney Cancer Organoids

Cancer constitutes the death cause in one of every seven deaths worldwide [118]. The tumor heterogeneity contributes to significant differences in tumor growth rate, tumor relapse, invasion ability, drug response and prognosis, and the establishment of preclinical cancer models that recapitulate inter- and intratumor heterogeneity is paradoxical to develop effective cancer treatments [119]. Cancer organoids – also termed tumoroids – are a promising platform for preclinical cancer research since they retain the heterogeneity of the original tumor, while maintaining a low cost and ease of use (Figure 12) [58], [118]. A major advantage of organoid technology for drug screening is that it allows generating simultaneously healthy and cancer organoids from the matching donors, thereby screening of compounds that specifically target the tumor tissue while leaving the healthy organoids unharmed is feasible [120]. Additionally, in contrast to patient-derived xenografts (PDTXs), cancer organoid technology is compatible with high-throughput assays. Similarly, as previously mentioned, organoids can also be engineered with state-of-the-art technologies, thereby normal organoids can be mutated into tumor organoids to decipher driver mutations of cancer initiation and tumor progression (Figure 12) [118]. Patient-derived tumor organoids can also serve to establish cancer organoid biobanks, which are repositories of a number of tumoroids from diverse cancer types. Cancer organoids can be extensively expanded, passaged and cryopreserved, as it occurs with cancer cell lines. Consequently, the establishment of cancer organoid biobanks would facilitate their immediate accessibility [121].

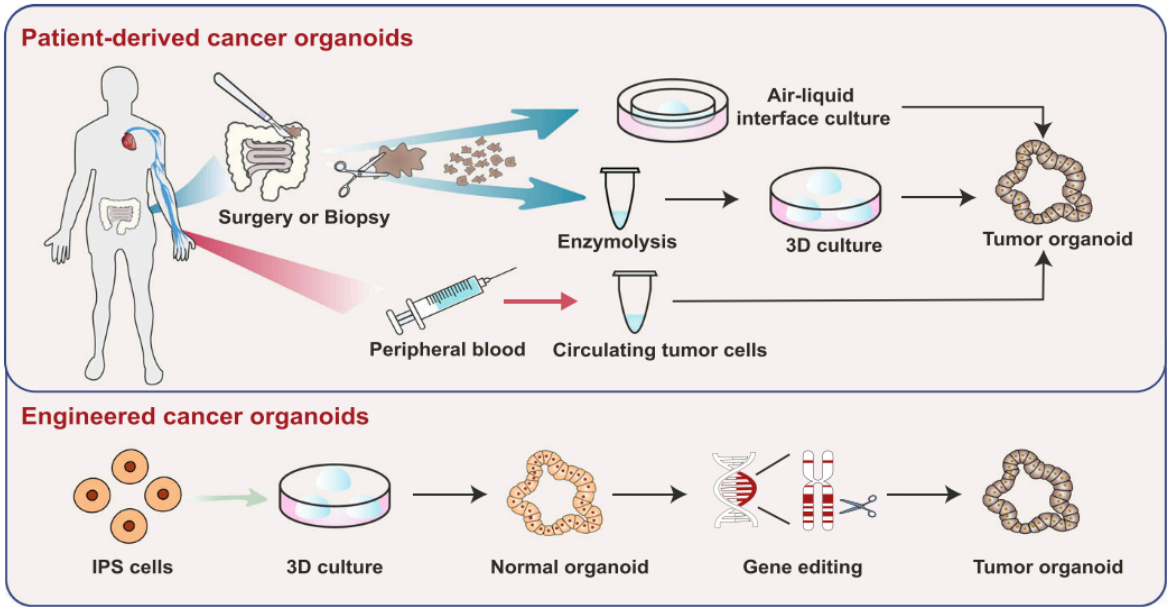

Figure 12: Approaches to generate cancer organoids. Patient-derived cancer organoids can be established from tissue biopsies or from circulating tumor cells. Alternatively, tumoroids can also be engineered using gene-editing techniques to dissect driver mutations of tumorigenesis. Adapted from [118].

As shown in Figure 12, patient-derived tumoroids can be established using both liquid biopsies that contain circulating tumor cells or from solid tumor biopsies. As a proof-of-principle of the applicability of patient-derived cancer organoids for cancer research, Broutier *et al.* [122] showed that primary liver cancer-derived organoids preserve the histological architecture, gene expression and genomic landscape of the original tumor, thereby making them a suitable platform to evaluate potential therapeutic agents for liver cancer. An alternative approach to study tumorigenesis is based on the combination of healthy organoids with CRISPR-Cas9 gene editing technology to dissect and identify recurrent mutations in the development of tumors [118]. As a proof-of-concept of this approach, Matano *et al.* [123] used gene editing technology to introduce recurrent mutations found in colorectal cancer in health intestinal organoids to elucidate the extent that these mutations contribute to human colorectal carcinogenesis.

Renal malignancies account for approximately 7% of all childhood cancers, being Wilms tumor the most common [124]. Drost and colleagues [124] have established the first organoid biobank of a number of kidney cancer types, including Wilms tumor, malignant rhabdoid tumors of the kidney or renal cell carcinoma. Interestingly, tumoroid lines typically showed a different phenotype from healthy kidney organoids established from tissue of the matching donor, which already indicates their tumor origin. In particular, Schutgens *et al.* [125] established and characterized tumoroid lines from Wilms tumor, also known as nephroblastoma. Wilms tumor arises from pluripotent embryonic renal precursors and it displays a characteristic tri-phasic histology that includes stroma, blastema and epithelium (Figure 14A) [126]. Tumor-derived organoids displayed a similar histological appearance to the original tumor tissue, including stroma, blastema and epithelium (Figure 14A) [125]. Additionally, whole-genome sequencing was used to evaluate the copy number variation (CNV) in the nephroblastoma. Interestingly, both the original Wilms tumor tissue and the tumoroid displayed typical CNVs that are associated with nephroblastoma, including loss of chromosome 16q and gains of 1q and of chromosomes 8 and 12 (Figure 14B) [125]. Collectively, these results indicate the tumoroids resemble the architecture of the original tissue and genetically reflect the tumor genome, thereby being a promising platform for drug screening using a personalized medicine approach since they might reveal patient-specific drug sensitivities.

Overall, cancer organoid models are a promising platform to accelerate cancer research due to their higher clinical relevance, but there are still gaps that need to be filled to improve their applicability. In particular, cancer organoids only contain epithelial cell types and progenitor cells, but they do not include non-parenchymal cell types (e.g. fibroblasts and ECs). In a similar manner, tumoroids are amenable to reconstitute tumors in a single organ, but they are not suitable to mimic multiorgan metastasis [118]. For this reason, the recent tendency is to couple cancer organoids with organ-on-a-chip to develop more sophisticated cancer models.

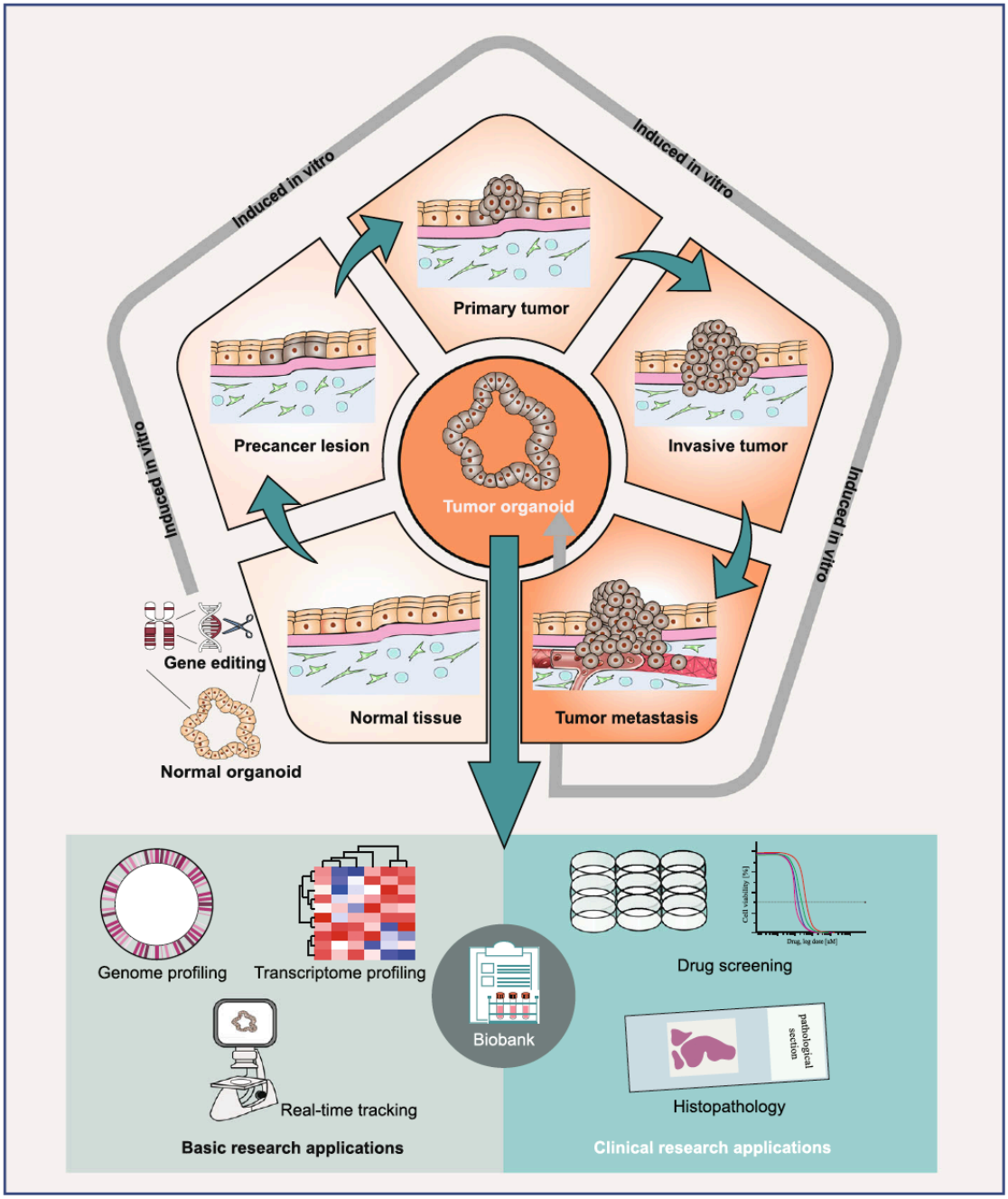

Figure 13: Overview of the applicability of cancer organoids for personalized medicine. Patient-derived tumoroids possess patient specific genetic and epigenetic contexts and they recapitulate histological architecture of the tumor. For this reason, tumoroids permit *in vitro* drug screening, thereby enabling personalized medicine. Additionally, as occurs with cell lines, cancer organoid lines can also be expanded and subsequently cryopreserved to generate organoid biobanks. Adapted from [118].

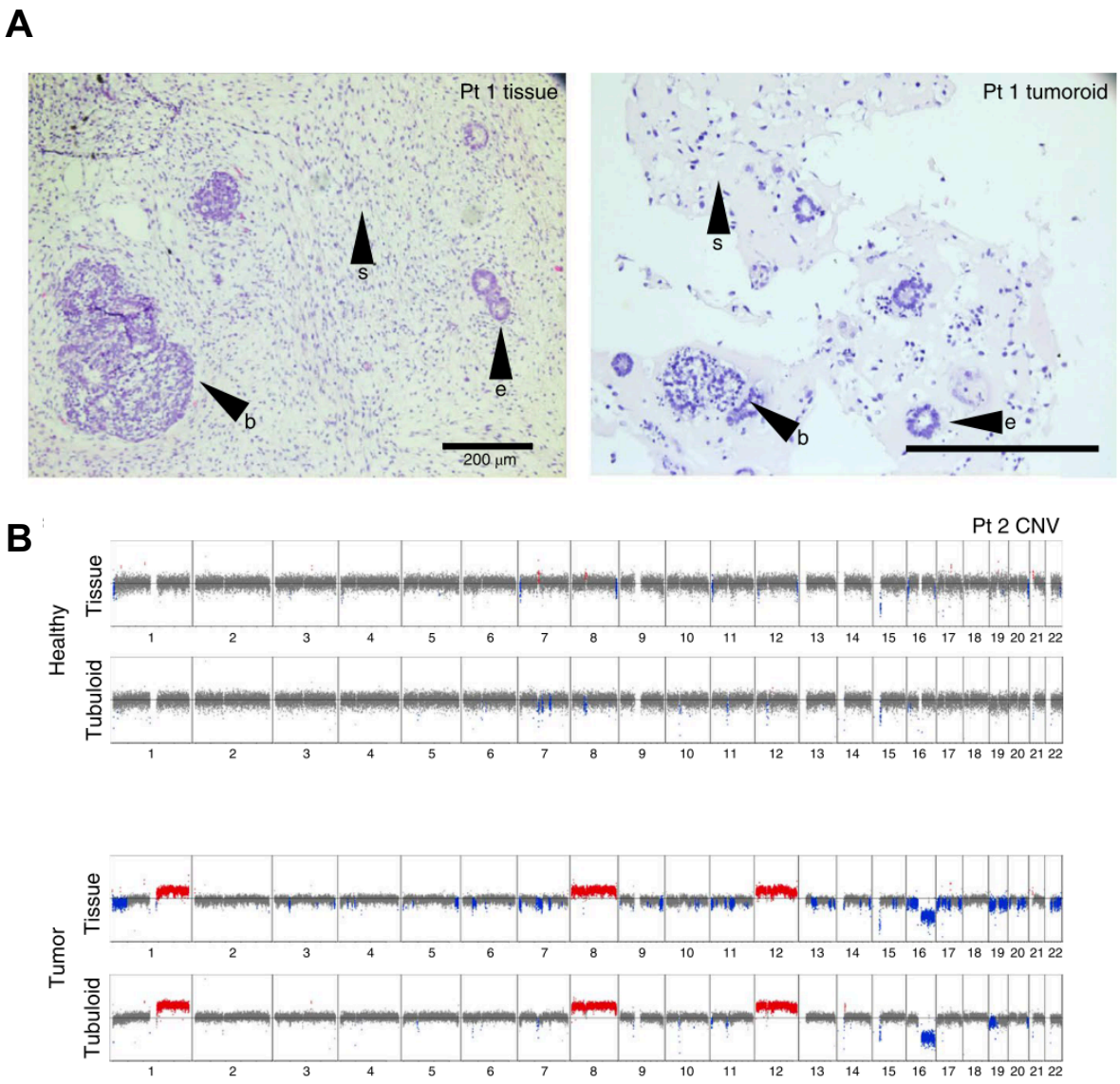

Figure 14: Tumoroid lines established from Wilms tumor tissue recapitulate the architecture of the original tissue and genetically reflect the primary tumor. A) Hematoxylin and eosin staining of the primary cancer tissue (left panel) and the respective tumoroid line (right panel). Both display the characteristic tri-phasic nephroblastoma histology, including epithelium (arrow head e), blastema (arrow head b) and stroma (arrow head s). **B)** Whole-genome sequencing of healthy kidney tissue and the healthy tubuloid line (upper panel) and of Wilms tumor tissue and tumoroid (lower panel). The healthy tissue and the respective tubuloid do not display copy number variations (CNVs) in their genome, whereas the tumor tissue and the tumoroid display typical Wilms tumor-associated CNVs, including 1q gain, gain of chromosome 8 and 12 and 16q loss. Adapted from [125].

3.3 Limitations of Kidney Organoids

Kidney organoids have already demonstrated a great potential for the applications indicated above; however, there are still many limitations to be addressed before the applicability of kidney organoids can be expanded, particularly aiming for a future clinical use for regenerative purposes.

The current limitations of kidney organoids are outlined in Figure 15. Overall, kidney organoids frequently contain non-renal cell populations (e.g. neuron and muscle cells) [127], suffer from batch-to-batch variability [128] and display limited cell maturation, since they resemble closer fetal tissues rather than adult ones [129]. Particularly, expression profiling has shown that kidney organoids show high similarity to the first semester human kidney [72]. Limited maturation may also suppose a safety concern due to the risk of tumor development from PSCs [130]. Additionally, the long-term immunogenicity elicited by organoids upon transplantation is still an enigma [57].

In particular, the presence of off-target cells, including neuronal and muscle cells, has been explained as a consequence of the way that directed differentiation protocols control the differentiation of mesodermal cells into specific cell fates [127], [131]. These protocols use morphogens that apply directional cues towards the primitive streak; however, different endpoints influence not only the different ratios of renal cells within the organoid, but also the presence of non-renal cell populations. For this reason, a better understanding of the lineage branching during differentiation is paradoxical to improve the current protocols to avoid the presence of off-target cells [131]. As a proof-of-principle, Wu *et al.* [127] reported that inhibition of brain-derived-neurotrophic factor, which promotes neuron survival and differentiation, reduces the presence of off-target neuronal cells within kidney organoids.

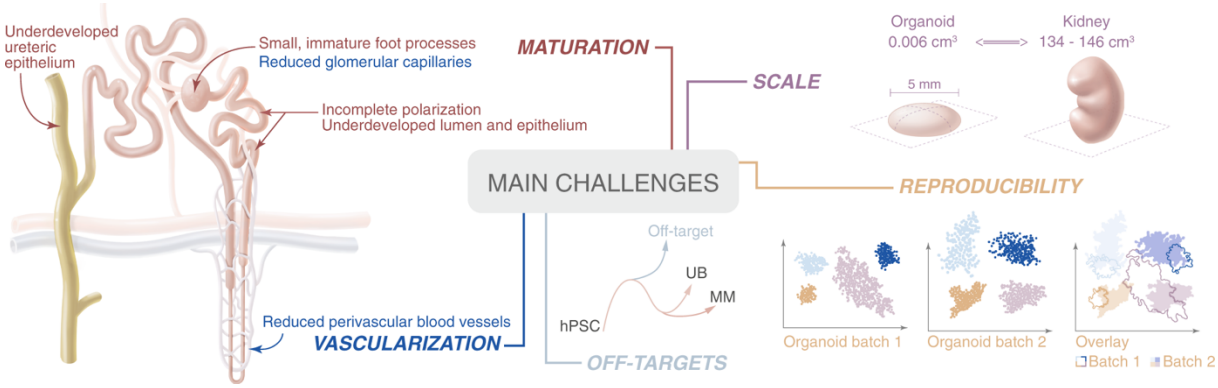

Figure 15: Kidney organoid challenges towards prospective use for regenerative medicine. Current established protocols are only capable of generating kidney organoids with an uncomplete maturation and a limited vascularization. Similarly, these protocols suffer from limitations in scalability and reproducibility. These limitations need to be addressed before any conceivable use of kidney organoids for replacement therapy for patients suffering from kidney disease. Adapted from [131].

In the present section, different approaches to overcome current limitations of kidney organoids for regenerative medicine use, including limited reproducibility, incomplete vascularization and the absence of the ureteric bud, will be discussed.

3.3.1 Next-Generation Kidney Organoids

Organoid reproducibility implies that, under the same experimental conditions, all derived organoids yield a similar organoid size, shape, cellular composition and 3D architecture [57]. The limited reproducibility of current organoid systems is considered a major bottleneck for their applicability, particularly in translational studies, such as drug screening [59]. Furthermore, in particular, when aiming to use organoids for drug testing and regenerative medicine, organoids are also required to be scalable and safe [57]. In this way, organoid establishment requires a supporting ECM that facilitates the formation of a 3D system. Matrigel, which is a natural ECM purified from Engelbreth-Holm-Swarm mouse sarcoma, is widely used as supporting matrix for organoid derivation [132]; however, its composition is variable and ill-defined and its animal origin supposes a limitation for the use of organoids for clinical applications due to the risks of immunogen and pathogen transfer [59], [133]. Consequently, establishing robust and reproducible organoid cultures using well-defined biomaterials is crucial for the use of organoids not only in regenerative medicine, but also in basic and applied research.

The inspiration from tissue engineering, which is a field mainly focused on the design of biocompatible materials to serve as cell-instructive scaffolds that restore human tissue or guide tissue regeneration, may further improve organoid cultures [59]. An overview of the different bioengineering approaches that are currently used in the new generation of organoids is outlined in Figure 16. Recent developments indicate that the combination of bioreactor technologies and the use of well-defined biomaterials provide more defined environments that increase the reproducibility and yield more physiologically relevant organoids [57]. Similarly, the coupling of organoids with microfluidics also hold the potential to deliver the morphogens that drive the directed differentiation in a more controlled manner, thereby also increasing their reproducibility [57]. As a matter of fact, on the usability of bioengineering approaches for organoids, Brandenger *et al.* [134] developed micro-engineered cell culture devices that allow the scalable and automated generation and real-time analysis of organoids trapped in microcavity arrays, which may facilitate the use of organoids in an industrial scale by increasing their reproducibility and standardization. Similarly, Qian *et al.* [135] used spinning bioreactors for the cost-efficient generation of large and reproducible PSC-derived brain organoids by the virtue of the improved diffusion of oxygen and nutrients that can be achieved in the bioreactor, which exemplifies the potential of the culture of organoids to standardize and scale-up their production.

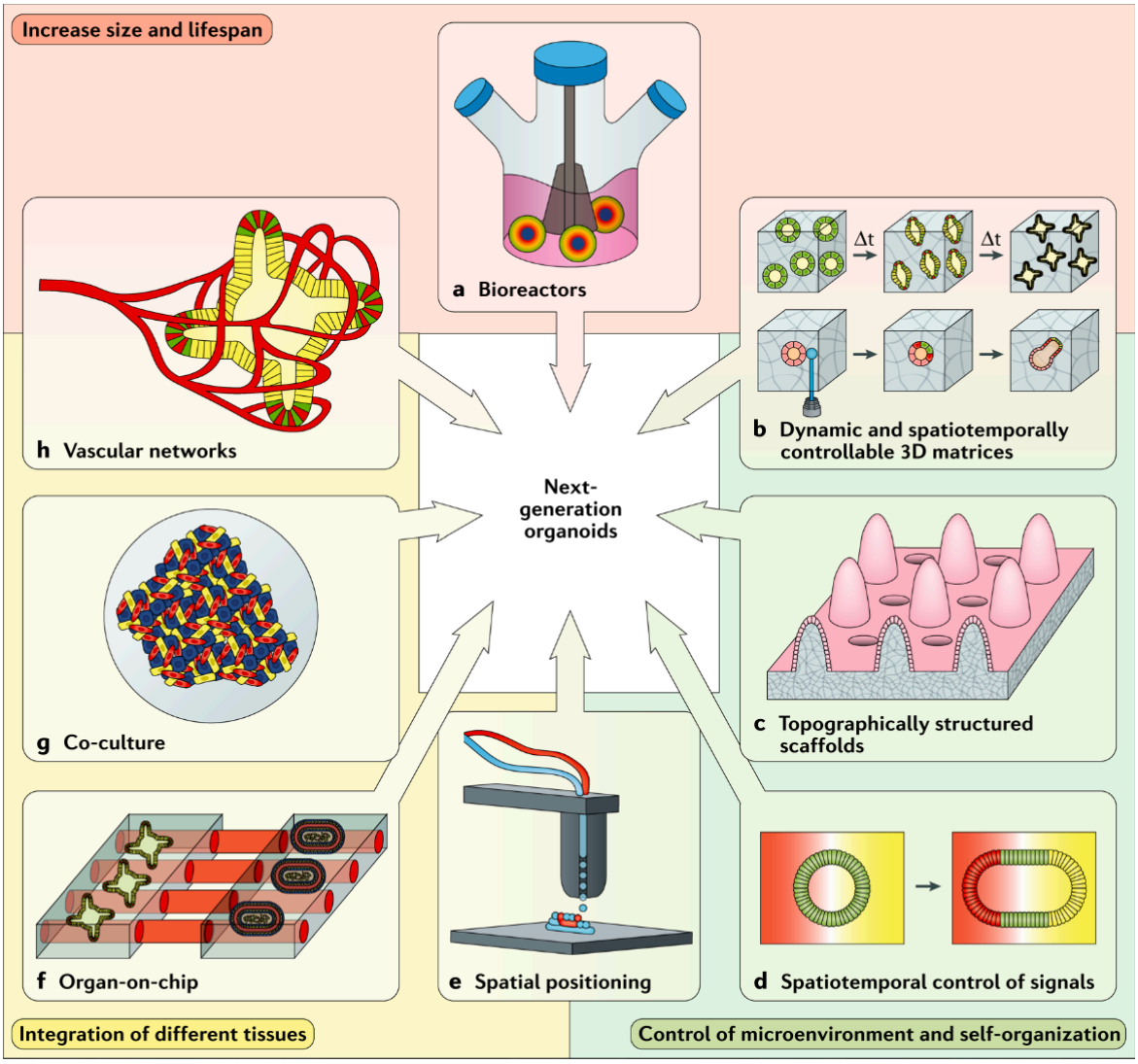

Figure 16: Bioengineering approaches to increase the reproducibility and applicability of existing organoids. Bioreactors **(a)** improve nutrient supply, which is a limiting factor when organoids grow in size. The use of dynamic and controlled extracellular environments **(b)** allows the self-organization of the organoids into the desired architecture. Similarly, micro-structured cell cultures **(c)** provide a manner to obtain topography that more closely resembles the original tissue. Mimicking the principles of the embryonic development, including a spatiotemporal control of the morphogens **(d)** that drive differentiation, might give rise to more physiologically relevant organoids. Additionally, the use of 3D printing to arrange a controlled spatial disposition **(e)** may increase the control over organoid self-organization. The integration of organoids into organ-on-a-chip technologies **(f)** serves not only to increase the organoid maturation *via* flow, but also to integrate organoids from different organs. The co-culture **(g)** of different cell types could be used to increase the complexity of organoids. The generation of a vasculature **(h)** within the organoid serves to increase the nutrient availability. Adapted from [59].

Single cell RNA-sequencing (sc-RNA-seq) is a powerful tool evaluate not only how accurately the cellular types identified in an organoid represent the targeted organ, but also to compare the divergencies between the different protocols used to establish the same type of organoid. To evaluate the reproducibility between different protocols to generate kidney organoids, Wu *et al.* [127] made a comparative study between the organoids produced following the Takasato [72] and Morizane [71] protocols. They revealed that both protocols yield a similar diversity of kidney cell types, but they vary in terms of cell ratio and differentiation state [127]. This finding may be helpful to orientate the kidney organoid protocol of choice depending on the scientific aim. Similar variations of cell proportions and nephron patterning have been observed even when using a single protocol and a single iPSC line [128]. Particularly, Phipson *et al.* [128] demonstrated that batch-to-batch variation is the major source of variability, whereas individual organoids from the same batch display high transcriptional correlation. To follow-up this study, Subramanian *et al.* [136] evaluated the faithfulness of kidney organoid protocols in different iPSC lines. They observed that kidney organoids from different iPSC lines are comparable, which serves as a proof-of-concept of the robustness and applicability of kidney organoids [136]. Different reasons may explain the batch-to-batch variability of kidney organoids, including disparities between batches of reagents, variations of PSC passages, which influences the pluripotency state at the induction of differentiation, or the technical variability between researchers [79].

The complexity of kidney organoid protocols has also been a limitation for its ease of use in high-throughput screening since current protocols cannot be automated and miniaturized. To address this limitation, Czerniecki *et al.* [137] established an automated and high-throughput compatible protocol, which was based on liquid handling robots, to culture kidney organoids. They reported that the high-throughput system increases the differentiation of kidney organoids [137] and it is a promising approach to reduce the batch-to-batch variability of kidney organoids. In particular, high-throughput-based systems hold promise for drug development and nephrotoxicity testing. Similarly, Higgins *et al.* [138] developed a bioprinted-based approach to generate highly reproducible kidney organoids.

Alternatively, the use of bioreactors to scale up the production of kidney organoids has also been explored. Przepiorski *et al.* [139] developed a spinner-flask biorreactor-based protocol to generate kidney organoids. Additionally, Kumar *et al.* [140] established a suspension culture method for the generation of kidney organoids. They reported that the suspension culture approach results in an approximately four-fold cell yield increase when compared to the conventional static protocols [140]. Remarkably, bioreactor-based approaches have shown to hold great potential to generate organoids in large quantities, while reducing the culturing costs [140].

3.3.2 Generation of Vascularized Kidney Organoids

Kidneys are highly vascularized organs and their adequate functionality involves the presence of incoming blood flow and the interaction with vascular ECs [84]. During the embryonic development, glomerular podocytes and mesangial cells develop closely with ECs, and their complete maturation as well as the development of the GFB are disturbed when ECs are absent [141], [142]. In adult kidneys, specialized ECs in the glomerulus, namely GECs, and peritubular capillaries play a major role in the glomerular filtration and tubular urine concentration, respectively [84]. Recently, using sc-RNA-seq, it has been reported that kidney organoids produced by the Morizane and the Takasato protocols contain only around 0.1% ECs (Figure 17) [143]. Consequently, a great concern for the translational applicability of kidney organoids for disease modelling and for regenerative medicine is that, although they contain some ECs, they lack a completely functional vascular network [84].

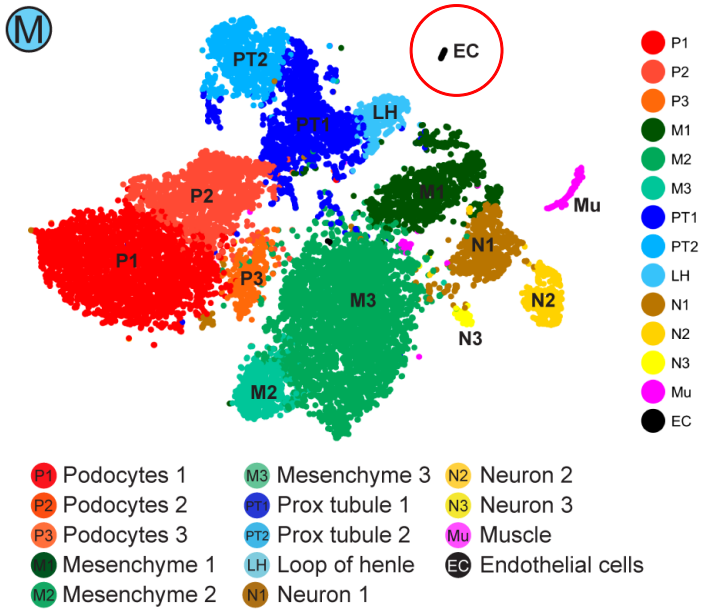

Figure 17: t-distributed stochastic neighbor embedding (t-SNE) plot of single cell RNA sequencing (sc-RNA-seq) of kidney organoids (day 26) established using the Morizane protocol. The detected clusters are indicated by different colors and the endothelial cluster appears highlighted by a red circle. Adapted from [127].

Different approaches have been used to increase the maturation and the development of an adequate vasculature within the kidney organoid. It has been shown that subcutaneous implantation of kidney organoids under the mice renal capsule hurdles the limited vascularization and it results in a significantly increased vascularization and maturation of glomerular structures [144], [145]. Similarly, this approach was also used by Wimmer *et al*.

[146] to generate functional vascular organoids [146]. However, the protocols that include animal transplantation to achieve a fully functional vascularization limit the translational scalability of these organoid models. For example, the need to generate human-animal chimeras to develop a complete organoid vasculature might have an influence on the disease pathophysiology, hence making them not fully suitable for human disease modelling [147]. Furthermore, the dependence on animal transplantation limits both scalability and translation of organoid-based approaches for regenerative medicine purposes [148].

On the other hand, it has also been demonstrated that *in vitro* exposure of kidney organoids to fluid flow stress enhances the maturation of nephrons, including the achievement of a functional vasculature. Recently, Homan *et al.* [148] used this approach by using a milifluidic culture system where kidney organoids were subjected to fluidic shear stress (Figure 18). They observed that shear stress exposure increases the number of ECs in kidney organoids. These ECs were able to form vascular networks containing lumens and, in some cases, invaded the glomerular structure [148].

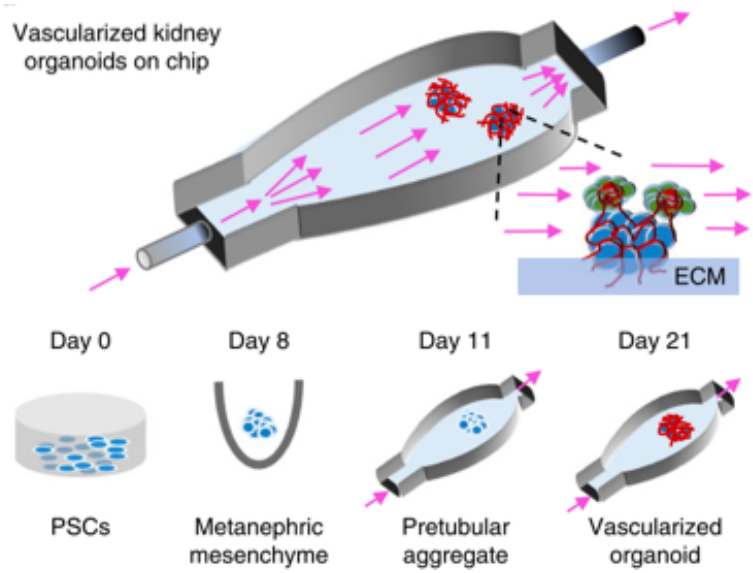

Figure 18: Scheme of the production of vascularized kidney organoids cultured *in vitro* under high fluid flow. Developing kidney organoids are fixed on an engineered extracellular matrix and subsequently placed within a perfusable milifluidic chip. Organoids are exposed to controlled fluid shear stress, which results in enhanced maturation and vascularization of the kidney organoids. Adapted from [148].

Similarly, Garreta *et al.* [95] recently reported that generating kidney organoids in soft hydrogel, which provides the adequate biophysical inductive cues, resulted in an increased maturation of kidney organoids, thereby resembling the second trimester fetal kidney. Additionally, in the same study, they also showed that transplantation of kidney organoids into a chick chorioallantoic membrane increases the vascularization of kidney organoids through invasion

into the organoid of chicken blood vessels [95]. However, unlike the milifluidic approach that increases the development of human blood vessels within kidney organoids [148], the emerging blood vessels in the ovo culture system are from chicken origin [95], which supposes a limitation for clinical use. Collectively, these studies highlight the major role of biophysical cues to increase the vascularization and the maturation of kidney organoids.

Producing *in vitro* vascularized glomeruli in kidney organoids supposes a major breakthrough for the future translational application of kidney organoids in regenerative medicine, but also to model kidney diseases where kidney vasculature plays a major role, such as the case of DN. However, it is still unclear to what degree the blood vessels generated using both *in vivo and in vitro* approaches to develop vascularized glomeruli possess and mimic the characteristics of the renal vasculature *in vivo* [84].

3.3.3 Higher-order Kidney Organoids for Transplantation

The use of kidney for renal replacement may require, apart from *in vivo* functionality, the presence of a urine collecting system that can be connected to the patient´s urinary system [80]. As previously discussed, the Morizane protocol [71] induces posterior IM differentiation to generate NPCs. For that reason, Morizane organoids contain only NPC-derived lineages (e.g. podocytes, Bowman´s capsules and tubular compartments); however, the absence of the anterior IM results in the subsequent absence of CD cells in the kidney organoid. Alternatively, Taguchi *et al.* [83] developed a protocol to separately generate both regions (posterior and anterior IM) to generate the UB and MM, respectively. Subsequently, through co-culture, both lineages are aggregated and self-organize to generate embryonic kidney-like structures that include differentiated nephrons and ureteric epithelium [83]. Additionally, to generate higher-order kidney organoids, the co-culture was also supplemented with mouse renal stroma [83].

Although these results were promising to generate kidney organoids that contain CD cells, none of the current kidney organoid protocols have been able to include a ureter that elongates from the kidney organoid [79]. Additionally, several concerns have been acknowledged concerning the nature of CD cells in the Taguchi protocol. Particularly, the authors defined CD as $GATA3^{+}/ECAD^{+}$ tubular structures; however, these markers are not only CD specific, but they are also present in distal tubules [149]. Additionally, CD structures generated using the Taguchi protocol differ from the characteristic highly organized branching structure of the CD tree in the adult kidney [84]. Consequently, future research will be required to discern the identity of the $GATA3^{+}/ECAD^{+}$ structures in kidney organoids generated following the Taguchi protocol.

4 Conclusions

The development of organoids has constituted a major breakthrough in 3D culture technologies. Organoids mimic human organogenesis “on-a-dish”, including early development and adult regenerative processes [57], and they resemble the original tissue while performing certain tissue functions, thereby organoids open up new avenues to study developmental biology and human pathophysiology. Particularly, patient-derived organoids bear the potential to bring the promise of personalized medicine to reality, since they can be used as patient-specific platforms for drug testing [120]. Consequently, we envisage a scenario whereby disease-specific organoids allow establishing the efficient therapeutic approach and window for that patient. Overall, the combination of the organoid approach with the state-of-art gene editing technology, and the advances in live imaging or biomaterials represent a tour de force that will have a great influence in the close future in our understanding of embryonic development and human diseases, which raises hopes for the development of novel therapies that improve life quality and expectancy.

In particular, kidney research has traditionally been hampered by the lack of suitable *in vitro* models that mimic the high complexity of human kidneys [84]. For this reason, kidney organoids constitute a platform that is already being used to gain understanding on renal pathophysiology [82], [103] and to screen for tubular nephrotoxicity [82], [100]. Although significant advances have been made in kidney organoid generation in the last five years, a number of shortcomings (e.g. vascularization, reproducibility, maturation) have to be overcome to make them suitable for clinical use. The combination of the organoid platform with engineering approaches (e.g. microfluidics, bioprinting and bioreactors) is promising to increase the physiological relevance of organoids [137], [139]. On the other hand, even though kidney organoids may be a game changer to overcome the kidney donor shortage, this approach is not achievable for clinical use in the foreseeable future.

List of Abbreviations (Chapter 2)

2D	2-dimensional
3D	3-dimensional
ACE2	Angiotensin-converting enzyme 2
ADPKD	Autosomal dominant polycystic kidney disease
AKI	Acute kidney injury
ASC	Adult stem cell
BMP	Bone morphogenic protein
Cas9	CRISPR-associated systems9
CD	Collecting duct
CKD	Chronic kidney disease
CM	Cap mesenchyme
CNV	Copy number variation
COVID-19	Coronavirus disease 2019
CRISPR	Clustered regularly interspaced short palindromic repeats
DN	Diabetic nephropathy
EC	Endothelial cell
ECM	Extracellular cell matrix
ESC	Embryonic stem cell
ESRD	End-stage-renal disease
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
GEC	Glomerular endothelial cell
GFB	Glomerular filtration barrier
HGF	Hepatocyte growth factor
hPSC	Human pluripotent stem cell
IGF	Insulin growth factor
IM	Intermediate mesoderm
iPSC	Induced pluripotent stem cell
Lgr5	Leucine-rich repeat-containing G protein-coupled receptor 5
MM	Metanephric mesenchyme
mRNA	Messenger RNA
NPC	Nephron progenitor cell
PA	Pretubular aggregate
PKD	Polycystic kidney disease
PSC	Pluripotent stem cell
PTDX	Patiend-derived xenograft

RA	Retinoic acid
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
sc-RNA-seq	Single cell RNA-sequencing
SIX2	Sine Oculis Homeobox Homolog 2
TF	Transcription factor
TGFβ	Transforming growth factor beta
UB	Ureteric bud
VEGF	Vascular endothelial growth factor
ZIKV	Zika virus

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